

nature

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Dream to reality in 25 years?

THE photocopier could hardly be called an unmixed blessing of 20th-century civilisation, but few would deny the substantial benefits it has conferred— or the number of jobs it has rendered unnecessary. Aware of the substantial cost (in foreign exchange) of photocopiers, the Indians set about designing one which could be produced by their domestic industry. In terms of the quality of copy delivered, the prototype devised at India's National Physical Laboratory in New Delhi, was as good as any model produced elsewhere. But, the innocent Western visitor asked, why is the loading of paper, the mounting of the material to be copied, the passage of exposed paper through developer still done by operators? Surely if you can apply high technology to the reproduction process, you can apply it to all other things going on in the machine. In our office the secretary just presses a button.

The reply to this rather insensitive question epitomises the dilemma of science and technology in the developing world—to make machines too automatic is to put people out of work, and the unemployed cannot look to a generous social welfare system to tide them over. Better a man employed on a modest wage doing a monotonous job; after all there is no shortage of manpower.

Such machines *versus* men, productivity *versus* employment issues have, of course, been raising themselves in a variety of guises all over the world for nearly two centuries, but this instance neatly illustrates the sort of clash in values that has endlessly to be resolved when developed technology encounters the developing world. These thoughts were occasioned by an important and little-reported lecture given, last week, by the Commonwealth Secretary-General, Mr Shridath S. Ramphal, former Attorney-General of Guyana, to the Science Policy Foundation in London.

Mr Ramphal's theme is that policymakers should be asking "what kind of science policy will contribute most to the eradication of poverty through a process of self-reliant economic advance that is consistent with social justice, environmental harmony and popular participation?" It is "appropriate technology" that should be at the centre of the development debate, not technology transfer. Technology, claims Mr Ramphal, is like genetic material in that it bears the code of social values of the society in which it was produced and sustained—export the technology to a region where the social values are

different and it may even prove counterproductive.

A science policy in the developing world that is closely allied to, indeed subservient to, social policy, is not without its problems, as Mr Ramphal recognises. There are bound to be many scientists whose interests could never be channelled in socially useful directions, and yet it would be disastrous to start a witch-hunt to hound all genuine intellectuals out and into the first job available in the developed world. The problem here is in differentiating between the distinguished thinker whose very presence at a particular university or laboratory raises the overall quality of the work done and students produced there, and the hanger-on who attempts Western-style research incoherently and inconsequentially (Mr Ramphal's words).

Another major difficulty is the small size of many developing countries. Sixty of them have populations of less than 5 million, so the rapid accumulation of a "minimum critical mass of scientific talent" committed to well defined social values is not going to be easy. Ideally, regional centres and policies could be evolved, but the scientist's famed supranational spirit often means in reality regular trips half way round the world rather than just over the border.

Nor, Mr Ramphal points out, can the developed world stand back and let the developing world try and work it out alone—"the internal values of the world scientific community cry out for change . . . development [should] be the substantive goal of the science policy of the industrial world".

Many will believe that the case made, while strong on idealism, is weak on realism. The scientific community, pushed into being a tool of social policy, might react in a thoroughly conservative way amid cries about pursuing excellence for its own sake and truth wherever it may lead. And yet evolution is occurring, particularly among young scientists, in their views on the functions of science. Provided the pace does not carry things too far towards revolution, and provided that ideologists are not allowed to make all the running, thereby deterring many excellent but apolitical scientists, there is a real chance that what today seems like an unlikely dream for the developing world could, by the year 2000 be an effective reality. The issue is worth wider discussion in the developed world than it has so far received. □



Silk purse for sows' ears?

Managing Director **W. Makinson** exposes the workings, triumphs, and failures of the UK National Research Development Corporation (NRDC)

DESPITE the occasional protestation to the contrary the university inventor is accorded an important and respected status among the NRDC's clients. Without his contributions it is doubtful whether Sir Stafford Cripps' concept of a statutory body for promoting technical innovation would have survived the political events of the late 1960s and early 1970s. During the corporation's life, however, the attitudes of central government and the universities to research and its industrial application have changed markedly. The declining status of Britain in international trade, and, in particular, the failure of its manufacturing industry to compete with those in other developed countries has underlined the need to promote and utilise university research. The creation of wealth is arguably as important as the pursuit of knowledge, even at the expense of traditional academic freedoms. The NRDC's task, as defined in the 1948 Development of Inventions Act, is to assist in the transfer of new technology from the laboratory to industry, and because the corporation aims to be self-financing it has adopted arrangements that provide wherever possible for the recovery of the costs incurred.

It is hardly surprising, therefore, that the role of the NRDC in universities has come in for some critical examination. Those affected by new constraints, and those strongly motivated by the wish for closer and more productive relationships between academic and industrial communities, retain a deep interest in the directions followed by the organisation. This reappraisal has led to suggestions that technology emerging from universities might be better exploited by other routes than those offered by the NRDC itself.

There could be grounds for this new attitude. Some inventors are industrially oriented and have enough familiarity with legal, patent and commercial matters to go it alone. But they are rare. Experience shows that there are proven advantages in adopting a professional, centralised approach to exploitation, particularly where new technology is of international significance. Successful exploitation requires a careful blend of talents and resources: professional skills, a wide knowledge of industry, financial resources, an ability to assess and take risks and, on occasion, some ruthlessness in decision

taking are all essential. This is often not appreciated by academic research workers, to whom the invention is the major event rather than the starting point of a process fraught with problems and pitfalls, many of which he is neither qualified nor equipped to meet alone.

Moreover, experience also indicates that the majority of inventions turn out to have little commercial potential, however professionally they may be handled. And, paradoxically, it is the almost quixotic willingness of the NRDC to devote time and money to university cases which probably do not justify support and consequently fail, that has led to criticism. This cannot be lightly brushed aside, however: the corporation needs to retain the confidence of the academic research community.

Patent protection

The NRDC plays a crucial role in both establishing and protecting industrial development that may arise out of basic research. The world of patents is now so complex and has so many ramifications that it cannot be effectively handled without the aid of the specialised professionals. Industry can, in some fields, get by without patent protection and can penetrate markets simply through dynamism, confidential know-how, and competitiveness. Where the element of innovation is high, however, companies usually insist on adequate patent protection for as long as possible after products become established. This is especially true in the case of, for example, pharmaceuticals.

The executive officers of the NRDC are well supported by commercial, legal and patent services on a scale which compares favourably with all but the largest multinational companies; its patent department, for instance, is among the largest in the United Kingdom. These expert advisory services are available to university inventors even if they are not already clients; the earlier an enquiry is made, however, the more useful any advice is likely to prove.

Once an inventor has placed himself in the hands of the NRDC he must accept certain limitations. He is less free to dictate the subsequent course of development and exploitation, while his share of any subsequent revenue is inevitably cut. This may conflict with a

drive for recognition through immediate publication; it may also preclude direct personal arrangements with individual companies, even though that could be the most obvious route to early exploitation. Existing or future patent rights are assigned to the NRDC, and equity is maintained through a revenue-sharing agreement which divides proceeds from licence royalties or other income between the corporation and the university concerned (or the inventor). Under current arrangements, the rights to inventions arising from projects funded by UK research councils have, in most cases, to be assigned to the NRDC anyway.

The time that the NRDC takes to decide whether support is worthwhile has occasionally been a source of frustration. But some delay is inevitable. Potential markets must be properly assessed, as must the potential patent strength. Regrettably, the more commercially attractive propositions are more likely to run up against established competition. But throughout, the corporation maintains close contact with the inventor.

Finance for development

Unlike foreign government agencies with similar responsibilities, the NRDC can provide funds not only for research programmes which may eventually prove of commercial significance, but can also support the further development of inventions already made. Indeed, if the support for further development were not available it is likely that many university inventions could not be effectively exploited. Bearing in mind the NRDC's reserve borrowing powers and currently favourable financial status, there is, within reason, no upper limit to funds available for any particular project, as long as it can be effectively deployed. The slogan adopted by the corporation in 1973: "£1,000,000 available for university research", is no idle statement.

The corporation always seeks the most effective route to commercialisation, so it usually attempts to involve an appropriate industrial organisation from the outset, either as a potential licensee and/or as a joint source of development funds. Negotiations may be carried out either by the university or the company, or both. It is quite normal for an inventor to act as a consultant on mutually acceptable terms.

Failure to arouse industrial interest in a particular project might often indicate that attempts at exploitation should be abandoned. On the other hand, it could be simply that a lack of sufficient evidence (in the form of a working prototype, for example, or of credible test results) is the only reason why industry cannot be persuaded that

an innovation is of importance—this is the “predevelopment gap” referred to in the Richards Report to the Engineering Board of the UK Science Research Council. While such a gap can be bridged by NRDC funds, the corporation will not shell out extra support merely to keep inventors happy; nor will it support an unnecessary research team, however competent. When further work is carried out in a university at the NRDC's cost a portion of any royalties is usually set aside to offset these expenses.

NRDC contributions to company development costs are recovered separately through a levy on subsequent sales of products exploited. The levy is calculated to provide a return reasonably related to the risks involved. The terms have from time to time been criticised as harsh, particularly when applied to proposals submitted by embryo, campus-based companies set up to exploit the results of research projects undertaken in a particular university. The criticism should be viewed against the statistical record, however, which shows that in three out of four cases the NRDC fails to recover its investment.

The corporation does not provide grants for company support, nor does it undertake to provide general working capital, except where an identified invention is involved. Companies must in such cases seek other sources of finance and accept the terms and conditions normally associated with them. It is worth noting, however, that support by the NRDC does not inhibit companies from qualifying simultaneously for other types of government assistance, nor need NRDC funding rank as a contingent liability where bank overdrafts or term loans are concerned.

Track record

Since its inception the NRDC has received about 5,000 proposals from universities, and taken assignment in about 2,000 cases. This compares favourably with the overall national picture including all sources of support—34,000 proposals and 6,000 assignments. So far, of the 2,000 university inventions supported by the NRDC about 200 have emerged as revenue earners; over the past five years this is about one out of every five or six submissions. The cumulative income attributable to university research is about £15 million, of which about £8 million has been recouped by the NRDC revenue-sharing agreements.

At present, the NRDC is involved in about 90 development projects involving universities, representing a total investment of about £1.5 million, about £1 million of which is actually being spent with the inventors. The largest single current commitment is that with

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The Dracone, a soft failure

the Southampton Institute of Sound and Vibration Research where £160,000 is devoted to research into techniques to produce light-weight quiet, diesel engines.

By far the most conspicuously profitable inventions so far have been those made 20 years ago by research workers in the Sir William Dunn School of Pathology at Oxford in collaboration with the Antibiotics Research Station of the Medical Research Council. The work involved led to the discovery of the drug Cephalosporin C and the subsequent isolation and identification of its nucleus; worldwide licensing arrangements currently provide approximately 80% of the corporation's total annual royalty income. Other significant ‘winners’ would include the anticoagulant Arvin (Penang and Oxford), portable heart/lung machines (Royal Postgraduate Medical School), peptides derived from bee venom (University College, London) and methods of extracting diosgenin (the starting material for steroids used in the contraceptive pill) from fenugreek (Nottingham). Perhaps the most notable engineering inventions were those of Manchester University relating to computers.

More recent developments for which high hopes are sustained include improvements in electrochemical cells (Newcastle), continuous counter-current ion-exchange techniques for uranium extraction (Imperial College, London), pioneering work on high modulus polymers, and novel flotation columns for mineral processing (Leeds), ultrasonics applied to metal-working (Aston), “surround” sound systems (Reading), speckle pattern comparators (Loughborough), and Pole Amplitude Modulation synchronous motors (Bristol). The NRDC has also set up a wholly-owned subsidiary company for the further development and marketing

of Genesys—a civil engineering computer-aided design system (CAD) initially developed at Loughborough University of Technology—and is in the process of creating a second software company for more generalised CAD packages. Though it is difficult to interpret statistics sensibly because of the often long gestation time of really successful inventions, the corporation is confident that the current input from university sources could maintain future royalties at a healthy level.

The NRDC has, of course, had its failures—sometimes spectacular—in its efforts to commercialise technically successful inventions. Any listing would have to include Dracones (large flexible floating containers for transporting fluids, based on proposals from Cambridge University) and the Tracked Hovercraft, which used the linear motor. Though the NRDC received no financial returns from either project both ‘failed soft’ in that the principles developed in these instances have been subsequently applied successfully to other industrial problems. Indeed, the Dracone, originally intended as an alternative to large oil tankers at the time of the Suez crisis, has been used to transport drinking water to the Greek islands, and might still play a useful role nearer home if the present drought persists.

Inevitably, many inventions from academic sources fail for simple technical reasons, usually in the translation from the original concept to practical realisation in commercial or industrial terms. Despite its years of experience, the corporation has no crystal ball; it will go on, no doubt producing more sows' ears than silk purses, but it has no intention of withholding support and encouragement wherever the rewards seem likely to justify effort and expense. □

USA

Treading softly on the ozone layer

A long-awaited report on the environmental effects of halocarbons was published in the United States last week. Colin Norman and Chris Sherwell report from Washington

THE US National Academy of Sciences (NAS) last week added its powerful voice to the scientific and political dispute about whether halocarbons, spewed into the atmosphere chiefly from aerosol spray cans, are causing serious damage to the Earth's ozone layer. The general press seemed to have some difficulty in deciding just what message the Academy was trying to convey, however. "Scientists Back New Aerosol Curbs to Protect Ozone in Atmosphere" ran the headline in the *New York Times*. "Aerosol Ban Opposed by Science Unit", said the *Washington Post*. Both were in fact correct, and the contrast reflects the finely balanced nature of the Academy's conclusions and recommendations.

The chief conclusion, set out in a long-awaited report by the NAS Committee on Impacts of Stratospheric Change, is that significant deterioration of the ozone layer will eventually occur if halocarbons continue to be released at their present rate. That would allow more ultraviolet radiation to reach the Earth's surface which, in turn would cause additional cases of skin cancer and other biological damage. Moreover, the committee warned of a possible effect on the world's climate from any continuing build-up of halocarbons in the atmosphere.

Alarming though such prospects may be, the committee cautions against taking precipitate action, such as banning the use of halocarbons as propellants in aerosol products. Instead, although it acknowledges that regulation is "almost certain to be necessary at some time and to some degree of completeness", the committee recommends that key uncertainties in the calculations should be cleared up before uses of halocarbons are restricted. The necessary information should be gathered in less than two years, the committee argues, and such a delay will cause little additional damage to the ozone layer.

The NAS recommendations were greeted with restrained enthusiasm by the halocarbon industry, and by the makers of hair sprays, deodorants and similar cosmetic products which together account for the major uses of the material. The industry-sponsored

Council on Atmospheric Sciences said in a statement last week, for example, that the report backs the industry's view that "more research is required before any national decision on the fluorocarbon issue can be reached". Some scientists and environmentalist groups were not so happy, however. A spokesman for the Natural Resources Defense Council noted, for example, that the NAS recommendation for a two-year delay in regulation "is a value judgment which scientists are no better equipped to make than anybody else". Clearly, publication of the report will not put an end to the controversy which has been raging over the ozone depletion theory for the past couple of years.

The theory was first advanced in 1974 by two scientists from the University of California, F. Sherwood Rowland and Mario J. Molina (*Nature*, **249**, 810; 1974). They argued, in short, that the very properties which make halocarbons so useful—their insolubility and inertness—also pose a problem: unlike most airborne pollutants, they are not washed out of the lower atmosphere by rain. They gradually drift up into the stratosphere, where they are broken down by sunlight, releasing free chlorine atoms which break down ozone molecules through a complex series of chain reactions.

If that sequence of events is actually taking place, the result would be a reduction in the concentration of ozone in the stratosphere, and a consequent increase in the amount of harsh ultraviolet radiation reaching the Earth's surface. The ultimate consequence would be an increase in the incidence of skin cancer, which is correlated with exposure to sunlight, and possible biological damage to plants and animals.

Publication of the theory ignited a scientific and political controversy as some scientists attacked it as being implausible, while others argued that it is frighteningly realistic. The implications for human health also led to suggestions that the use of aerosol sprays be curbed, and prompted a massive research effort to test the validity of the theory.

Then, earlier this year, yet another alarming theory was put forward, namely, that accumulation of halocarbons in the atmosphere will result in a so-called "greenhouse effect", increasing the Earth's temperature and perhaps altering its climate.

The NAS committee has thus been poring over a mass of information accumulated since Rowland and Molina

published their theory, and its report has been delayed for nearly six months by the appearance of important and conflicting pieces of data. In the end, however, the committee concluded that the evidence produced so far supports the ozone depletion theory. According to the committee's Chairman, John Tukey, a statistician from Princeton University and Bell Laboratories, the mechanism is now "a relatively well understood process".

It should be noted at this point, however, that it is impossible to test the theory by direct measurements of ozone concentration in the stratosphere since available measuring techniques are too insensitive to detect relatively small, long-term variations in the ozone layer. The theory is therefore based on laboratory experiments and computer analyses, supplemented with direct measurements of the atmospheric concentration of various key links in the chain reaction, such as chlorine, hydrogen chloride and so on. The whole business is a little like building up a jigsaw puzzle from badly cut and incomplete pieces.

Nevertheless, the committee has concluded that, if halocarbons continue to be spewed into the atmosphere at the rate they were released in 1973, the ultimate effect would be to reduce the concentration of ozone in the stratosphere by about 7%. It will take several decades to reach that level of depletion, however, since once released into the atmosphere, halocarbons can take up to a century to reach the stratosphere to do their work.

The figure of about 7% is deceptively precise. In fact, Tukey was careful to point out last week that it may turn out to be as low as 2% or as high as 20%, the uncertainties being caused by the complexity of the reactions—as many as 30 or 40 may be involved—and by the incompleteness of the information so far accumulated. That's one reason why the committee recommended against an immediate ban on non-essential uses in aerosols and suggested that more data are required.

Even if all release of halocarbons were to cease immediately, the committee has calculated that the material already in the lower atmosphere would continue to rise into the stratosphere and increase the destruction of the ozone layer for at least a decade. The ozone layer would then recover very slowly, with only half of the reduction being restored in 50 years. As for the consequences of delaying regulation by two years, the committee has calculated that if the figure of 7% reduction is correct, "whether a halving in (halocarbon) use and release were to take place in 1977 or 1979 would alter the

ozone reduction at any later date by no more than 1/6 per cent". In other words, "costs of delay in decision are small, not more than a fraction of a percent change in ozone depletion for a couple of years' delay".

Some perspective on the uncertainties in these calculations can be gained from a brief look at the problems which the committee encountered in putting out its report. The report was originally scheduled for publication in April but just as it was being put together, some new information, which seemed to throw the whole basis of the ozone depletion theory out of the window, suddenly came to light. In short, the theory would fall apart if there is a natural process which ties up chlorine atoms before they can attack the ozone molecules in the stratosphere. It seemed for a few weeks that just such a mechanism had been discovered. The suggested mechanism was the formation of chlorine nitrate, a compound which may be relatively stable under stratospheric conditions from chlorine atoms liberated from halocarbons and oxides of nitrogen already in the stratosphere.

When calculated rates of formation of chlorine nitrate were slotted into the equations, it turned out that halocarbons may even have a positive effect on the ozone layer—in other words, they may increase ozone concentration by removing oxides of nitrogen from the stratosphere. Later computations indicated, however, that the assumed rate of formation of chlorine nitrate had been greatly overestimated, and the effect of the mechanism is actually quite small.

As for the potential climatic effects of halocarbons, the committee suggests that precise information is equally hard to come by. Nevertheless, its best guess is that there will be a warming effect due to absorption of infrared radiation by halocarbons in the atmosphere, the effect being similar to that proposed for carbon dioxide accumulated through the burning of fossil fuels. Tukey suggested that the effect from halocarbons in the year 2000 might be about 40% of that due to burning fossil fuels, but he added that the relationship between such warming trends and broader climate changes is very poorly understood. "There are going to be some climate effects and it would be, I think, a disservice to put hard numbers on them this year", he said.

Although the committee recommends against immediate restrictions on the use and release of halocarbons, it does urge a number of steps to prepare the way for regulation should it be needed. At present, the authority to regulate the use of aerosol sprays is vested in at least three different agencies—the Food and Drug Adminis-

tration (FDA) the Environmental Protection Agency (EPA) and the Consumer Product Safety Commission (CPSC)—and it is questionable whether any of them really has a powerful enough mandate to act. Legislation is clearly required. Last week, by coincidence, Congress finally approved the Toxic Substances Control Act, a legislative landmark which, according to some interpretations, may provide EPA with enforceable authority over halocarbons. But the Bill is opposed by industry and may be vetoed by President Ford. Similarly, Congress is close to passing a series of amendments to the Clean Air Act, which again would give EPA a powerful lever to regulate aerosol products. The problem here, however, is that the House and Senate may not be able to settle some basic disagreements on other parts of the Bill before the October 2 recess. Thus, legislation may have to wait until next year.

The committee does, however, recommend one potentially important step which could be implemented relatively quickly. It suggests that aerosol sprays containing halocarbon propellants should bear a label stating that fact. The FDA, which has authority to regulate cosmetics, probably has the power to enforce such labelling. Though the committee argues that

"labeling should in no sense be regarded as a substitute for regulation but rather as an aid to consumer self-restraint", the effect could be rather large, since consumers are already beginning to turn away from some aerosol products. In 1975, for example, the use of halocarbons declined by 15%—the first decline in more than a decade (the accompanying table shows 1975 figures).

As for the effects on industry of a ban, the committee says they would be "appreciable". Just how appreciable can be gleaned from the fact that total world production of the two halocarbons with which the committee is concerned—namely, CFCI_3 or F-11, and CF_2Cl_2 or F-12—amounted in 1974 to nearly one million tonnes. But, says the committee, the industrial consequences do "not loom large" against the background of "a possible, although very small, change in world climate".

Finally, it should be noted that the US accounts for almost half the total world use of halocarbons. Thus, if the US Government decides on regulation, it would only attack half of the problem. The committee therefore recommends that other countries should be encouraged "by whatever appropriate means are likely to be effective" to take similar action. □

Estimated worldwide releases of F-11 and F-12 in 1975 (millions of pounds)

Aerosols 1115.1 (74.5%)	Personal	Antiperspirants/deodorants	458.4
		Hair care	401.5
		Medicinal	37.3
		Fragrances	2.3
		Shave lathers	0.9
		Others	34.4
	Household	Room deodorants	17.7
		Cleaners	9.6
		Laundry products	23.4
		Waxes and polishes	9.2
		Others	9.2
	Miscellaneous	Insecticides	33.3
		Coatings	22.9
		Industrial	39.0
		Automotive	8.0
Vet. and Pet		2.3	
Others		5.7	
Air conditioning refrigeration 204.7 (13.7%)	Mobile air conditioning	89.8	
	Chillers	42.9	
	Food store	33.1	
	Beverage coolers	5.8	
	Home refrigerators and freezers	5.8	
	Miscellaneous	27.3	
Plastic foams 176.5 (11.8%)	Open cell	100.0	
	Closed cell	76.5	
Total			1496.3

Figures are based on the annual incremental releases of F-11 and F-12 indicated for 1975 in a 1976 report by the Manufacturing Chemists Association, and the detailed percentage analysis by users in the US for 1973 in a 1975 report by Arthur D. Little, Inc. for the EPA. Figures do not include USSR and Eastern Europe.

Breakdown between F-11 and F-12 not available. If F-11(CFCI_3 , 77% chlorine by weight) could be replaced by an equal weight of F-12 (CF_2Cl_2 , 59% chlorine by weight)—not often feasible—the amount of ozone reduction would be decreased.

Source: *Halocarbons: Environmental Effects of Chlorofluoromethane Release*, Chapter 1 and Appendix D.

BRITAIN

Recipe for less disaster

The first ever report of the recently formed UK Advisory Committee on Major Hazards (CMH), which recommends improved safety measures for potentially hazardous industrial installations, was published last week. Alastair Hay reports

THE new measures advocated in the CMH report* could provide a framework for the safer building and siting of industrial plants in Britain. The report considers the hazards of "explosion, the sudden release of toxic substances (and) cataclysmic fire", and industries that would come under closer scrutiny include those producing fertilisers, explosives, petrochemicals and nylon and other polymers. Nuclear installations are not considered in the report.

The report is the first of its kind to be produced as a result of a government commission in any country. The CMH was appointed by the Health and Safety Commission (HSC) following the disaster at the Nypro chemical plant at Flixborough in June 1974, which killed 28 people, injured more than 100, and resulted in an estimated £28 million worth of damage to the immediately surrounding area. The Flixborough explosion was the most devastating in Britain since the end of World War II.

Prominent among the report's 38 recommendations is a call for a notification scheme that would allow the UK Health and Safety Commission (HSC) to identify particularly hazardous installations. In drawing up a list of notifiable installations the committee identifies 8 different types of plant, but ignores nuclear installations, in line with its original brief. An estimated 1,000-3,000 sites at present operating within the UK would fall within the proposed list, of which at least one in ten is operating to safety standards giving possible cause for concern, the report suggests. Moreover, while potentially notifiable plants are distributed throughout the UK, the report identifies specific areas of concentrated development such as Teesside and Canvey Island where a chain reaction of explosions might pose an additional hazard. The proposed list of notifiable installations includes:

- those storing or processing toxic material where there could be an emission of toxic gases or vapours equivalent in effect to more than 10 tonnes of chlorine.

- those storing or processing flammable materials where there could be a rapid emission of more than 15 tonnes of flammable gases or vapours.

- Those storing or processing more than 5 tonnes of materials which are intrinsically unstable or of very high exothermic reactivity. Examples are ethylene oxide, acetylenes, and organic peroxides.

- Those with a large inventory of stored pressure energy, operating at 100 bars or more, using gas phase reactions.

- Those storing or processing more than 10,000 tonnes of flammable materials with a flashpoint of less than 22.8 °C.

- Those storing or processing more than 135 tonnes of liquid oxygen.

- Those storing or processing more than 5,000 tonnes of ammonium nitrate.

- Those storing or processing materials which during a fire could cause an emission of toxic gases or vapours equivalent in effect to more than 10 tonnes of chlorine.

The list would apply to both existing and proposed installations, though the CMH has stressed that it is only provisional, and that a future list will almost certainly be wider ranging.

The report underlines the increasing importance of getting design and operating procedures right the first time. "Because of their present-day size, and throughput," the report says, "there are now many plants throughout the world where a critical first mistake can result in disaster". Moreover, the committee warns of the increased dangers to local communities: the report notes that while "the probability that an individual worker will be involved in a fatal accident has notably fallen, the chances [of] a plant failure . . . involving the public at large . . . [have] become considerably greater".

Some of the specific proposals included in the report stem directly from recommendations made by the court of inquiry into the Flixborough disaster. For instance, the report advocates that industrial pipework, valves and pumps, at present often only tested before assembly, should also be tested following installation, when they form part of a total pressure system. It also recommends that any changes in the operating procedure at plants should be reported to the HSC for evaluation. Control rooms—that at Flixborough, built of brick, was completely destroyed in the explosion—should be single storey, reinforced concrete buildings, situated in a relatively safe area, while black box recorders similar to those used in air-

craft, should be introduced to monitor plant operations, allowing the identification of fault sequences leading to accidents. Moreover, safety officers should have easy access to international data banks on industrial accidents and plant safety features, the report says.

The report advocates closer consultations between local authorities and the HSC when planning permission for new industrial plants is being considered. Neighbouring authorities likely to be affected by final decisions should also be included in the discussions. But the CMH has not yet considered the consequences of closing down potentially hazardous sites already in operation. At present, the compulsory closure of existing plant involves local authorities in the payment of considerable sums in compensation. The committee plans to discuss this problem with the Department of the Environment.

The Report does not advocate the enforced notification of 'near misses', which, it feels, could inhibit the flow of information. Mr James Tyc, director of the British Safety Council, welcoming the report, estimated that for every fatal accident in British Industry, there are three involving injury to workers, thirty resulting in damage to plant, and six hundred 'near misses'. Nonetheless, both industry and the HSC recognise that the lessons to be learned from a 'near miss' are as valid as those gained in the wake of a full-scale accident.

Professor Bryan Harvey, Chairman of the CMH, stressed the responsibility of management in introducing appropriate safety measures even before they became compulsory. Industry, he said, would in future have to give far more consideration to safety factors. Speaking for the chemical industry, Mr Donald Bennett, Chairman of the Chemical Industry Safety and Health Council, welcomed the report as "broadly consistent with the industry's own thinking on the form of control required". But he singled out areas needing further investigation, among them "the definition of the unit of notification", what parts of plants, processes or sites should be licenced, and the problem of selecting "truly independent experts" to act as arbitrators between industry and the HSC.

Mr Bennet's sentiments are not inconsistent with those of the CMH itself, outlined in the introduction to the report. The committee acknowledges the need for further discussion and research, and admits to the preliminary nature of much of the work completed so far; the report has been distributed to various interested organisations with requests for specific comments on the committee's proposals. □

*Advisory Committee on Major Hazards. First report of the Health and Safety Executive. HMSO, £1.00.

BRAZIL

CONCERN for the environment is increasing in Brazil, a country which until recently dismissed ecological pre-occupations as detrimental to industrial development. But even though public consciousness about the environment in this vast nation of 110 million people has been raised, little has been achieved in practical terms, and the environmental battle is far from won.

On the plus side, however, a new Presidential-level, environmental protection office—known as SEMA—has been authorised to veto state financing of industrial undertakings lacking adequate pollution safeguards. The head of SEMA, Paulo Nogueira Neto, regards this as a major victory for the environmental protection movement. And he does not believe it will be very hard to persuade Brazilian industry to fight pollution before producing it. "The inclusion of anti-pollution equipment in an industrial project usually adds only 2% to the project's total cost", Nogueira Neto declared recently. "On the other hand, if a factory is ordered to install antipollution equipment after it already has gone into operation, the cost of such equipment will be much higher." (There's a major catch to this, however: SEMA does not yet have adequate authority to shut down existing transgressors, and cannot force them to install antipollution gear.)

Also on the plus side, the Federal Government's much criticised and undermanned Forest Service has scored some recent successes in catching clandestine hunters, and in preventing the export of hides of illegally slaughtered protected species such as deer, monkeys and alligators. The Forest Service plans to burn 100,000 recently apprehended pelts and skins, hoping to deter future unlicensed hunting. Most illegal hunting takes place in Mato Grosso State and the Amazon Jungle, which, because of their immense size and lack of roads and communications, are practically impossible to patrol.

The Forest Service has also won a victory of sorts by successfully fining the Brazilian subsidiary of Volkswagenwerk AG the equivalent of \$25,000. Volkswagen has gone into the cattle ranching business in the Amazon Jungle, taking advantage of liberal Brazilian tax incentives. The fine follows a technical violation concerning the clearing of trees on Volkswagen's property. The Forest Service at first wanted to fine Volkswagen the equivalent of more than \$5 million for the alleged un-

authorised felling of some 9 million trees, but the plan was reportedly shot down at higher levels within the government, which wants to maintain a favourable climate for foreign investment.

On the minus side of the environmental picture, nearly 2,000 residents of a slum district in the north-eastern city of Salvador were hospitalised after breathing potentially deadly chlorine gas from a chemical plant. The plant is an indirect subsidiary of Petrobras, the state oil company, and will probably escape with only a fine.



Salvador is, in fact, apparently a major target of industrial polluters. The neighbouring Atlantic Ocean and several rivers in and near the city are becoming poisonous repositories of mercury, cadmium, sulphuric acid and ferrous sulphate. Jun Ui, a Japanese expert, has studied the situation in Salvador and concluded that the population is on the verge of mass poisoning, similar to the mercury poisoning that caused a scandal in Minamata, Japan, during the 1950s. Professor Ui has found traces of mercury in the hair of Salvador residents, and has said that cadmium in the region could cause bone disease.

People in several Brazilian cities have begun to band together to agitate for the preservation of urban green space; so far, however, none of these groups have been very effective. In Sao Leopoldo, a suburb of Porto Alegre, schoolchildren recently held a public demonstration against the felling of stately palm trees within the school grounds. The Forest Service fined the school administration and ordered it to stop, but not before 25 coconut palms had fallen to the chain saw. In Belo Horizonte, a growing city of 1.5 million people that is rapidly turning into a "concrete jungle", citizens meanwhile took to the streets to protest against the felling of a grove of trees on Church land.

But the Church obtained permission to continue the clearance, and now plans to build a shopping centre and office building on the newly stripped site.

● An antismoking lobby has emerged in Brazil. The Rio Grande do Sul State Medical Association has managed to scrape up the equivalent of \$40,000 for a three-month advertising campaign warning of the health hazards of smoking. It aims to persuade the Brazilian news media to carry antismoking propaganda to offset cigarette advertising. There is also pressure for government health warnings on cigarette packages and tobacco advertisements. The anti-smokers face powerful adversaries. Brazil's cigarette industry, dominated by a subsidiary of the giant British-American Tobacco Company, is one of the biggest advertisers in the Brazilian news media, and pays more than \$1,500 million in Brazilian taxes each year.

● Brazilian petroleum engineers have always believed that there is oil in Brazil's Amazon region, a belief not generally shared by the world's oil industries. Now, however, Petrobras has found oil off the coast of the far northern territory of Amapa, and along the continental shelf off Rio de Janeiro State. It is still too early to tell how much oil may be in the area of the test-well but the fact that Brazil has finally discovered Amazonian oil is important in itself. Brazil, whose principal domestic source of oil is the north-eastern state of Bahia, still imports 80% of its annual oil requirements, which causes serious balance-of-payments problems. Five international oil corporations plan limited prospecting operations in Brazil.

● A Brazilian obstetrician is encouraging his patients in the prosperous middle-class city of Curitiba, in the southern part of the country, to have their babies in the same way as the native Indians: squatting instead of lying down. Dr Moises Paciornik has studied primitive Indian tribes in Brazil and has concluded that squatting births lead to less pain and fewer postnatal complications. Once contractions have started, delivery is safer and quicker, if gravity is allowed to help, Dr Paciornik says. He has adapted one of the delivery rooms in his Curitiba clinic for squatting births, complete with an Indian-style mat on the floor, and he says his urban patients show surprisingly little reluctance about giving birth the Indian way.

Bruce Handler

IN BRIEF

Electric cars for US

Congress last week won a small, but important, skirmish with the Ford administration over a plan to develop an electric car industry in the US. It overrode a Presidential veto on a Bill to devote up to \$160 million to research, development and demonstration of electric car technology over the next six years. The key part of the Bill—and the part which stuck in Mr Ford's throat—is a scheme to create a market for electric vehicles through direct government support. In the next three years, the Government is empowered to purchase up to 2,500 electric vehicles, with a further 5,000 within five years. Mr Ford had vetoed the legislation feeling that the technology was not sufficiently advanced for a subsidised purchasing programme of that size.

Engineers building up

For all that is being said about the sad state of recruitment into engineering, figures just released by the Universities Central Council on Admissions (UCCA) show a steady growth in the number of applicants, and admissions, to universities for engineering courses (Statistical Supplement to the Thirteenth Report, 1974–5, UCCA, £1.25). Since 1973 electrical civil and mechanical engineering have each registered gains in applicants—firm figures for 1975, and reliable forecasts for 1976 show that in three years engineering has gained 30% more applicants. Mathematics, physics and chemistry remain in the doldrums; chemistry was a few per cent up both in 1975 and 1976, but physics dropped several per cent in 1975 and just held its ground in 1976.

Soyuz in orbit

Carrying photographic equipment developed partly by the East German company Carl Zeiss (Jena), the latest Soviet space-shot, Soyuz 22, went into orbit last week. Special emphasis is being paid to the photography of large tracts of Soviet territory, and while practical results are anticipated—similar, it is hoped, to those from the Soyuz 12 survey which revealed potential oil- and gas-bearing zones in the region of the Mangyshlak and Buzachi peninsulas—the main aim is to develop new techniques. Additionally, as a follow-up to earlier experiments during which cultures of *Proteus* bacteria grown in space showed signs of developmental deviations, experiments aboard Soyuz 22 have been designed to investigate the phenomenon.

SCIENTIFIC meetings used to be almost routinely enlivened by projectionists who inserted slides upside down, backwards, and so on. But the introduction of carousel projectors has largely done away with this form of amusement, especially when the lecturer is artful enough to load the slides in advance. I always try to meet projectionists ahead of my talks. Most of them are males, often students who take the job as part-time employment. I usually scrutinise the projection booth for copies of *Sports Illustrated* and soft-core porn magazines, which I scan briefly to soothe my nerves. If present, such reading matter shows that the projectionist will not be listening for slide-changing cues during my lecture, and I know that I must shout my requests.

One of my adventures as a lecturer had an almost dream-like quality. It was in Las Vegas. I arrived at the "meeting hotel" to speak to a convention of dietitians. After threading my way past slot machines and blackjack tables, I found that the theme of the day was "Nutrition in the Age of Aquarius". The delegates wore emblems proclaiming their zodiacal signs. Some of them were multiparous married women whose badges stated "I am a Virgo", which seemed incongruous to me. The slide projector had somehow been overlooked, and it was one hour to launch time. A hotel employee was delegated to drive me on a hunt for the machine. We bowled along a road that headed west, past shacks, into the relentless desert landscape until I realized that we were well on the way to Death Valley. Eventually we backtracked and spotted a Quonset hut that yielded a pro-

jector, and we got back to the casino in time. I was so stimulated by the experience that I preceded my lecture with an impromptu summary of my unvarnished opinion of the zodiac and of horoscopes.

Previously in these pages, I have described the incident in which

Slide slips



THOMAS H. JUKES

Marianne Grunberg-Manago disappeared from sight at the start of her lecture, in a manner similar to the descent of Erda in *Das Rheingold*. I made a mistake; I have received a peremptory letter of reprimand from the head official of the American Society of Biological Chemists, pointing out that it happened in Philadelphia in 1958 rather than in Atlantic City in 1963. Another correspondent, more sympathetic, says that he re-

members that Marianne blew kisses and waved to the audience as the platform went down. Be that as it may, I recall another Federation Meeting—I think it was in Chicago—addressed by Larry Irving, in which some interesting slides were shown at a long lecture on respiratory physiology. They were remarkable for all being projected sideways. The large audience developed a transient form of torticollis resembling a sort of a lateral whiplash injury. I believe that the explanation for the misoriented slides had a nationalistic background. It seems that British slides used to be 3½" square, American slides 3½"×4", while the Canadians, with their altogether admirable trait of self-assertiveness, used slides that were 4" square. The slides in question must have been prepared by a Canadian for showing in US projectors, which the Canadian evidently thought received 3½"×4" slides vertically rather than horizontally.

One of the new amenities is an antenna that is coiled around the lecturer like the string around a parcel. I cannot vouch for the following story that was related to me, but even if it didn't happen, it has obvious possibilities for users of Citizens Band radios. An unfortunate scientist swathed in his antenna, switched on the microphone and started his talk. Or tried to. But instead of the lecturer's voice the equipment picked up, on the same wavelength, the thunderings of a fire-and-brimstone preacher at a nearby revival meeting. I am told that the scientist plucked the offending antenna from his body in a vain effort to quell the interruption.

news and views

X-ray cryoenzymology

from C. C. F. Blake

ALTHOUGH the degree of knowledge constituting an "understanding" of the catalytic power of enzymes may be a matter for debate, the basic nature of certain kinds of information is not in dispute. These essential pieces of information include the three-dimensional structure of the enzyme, and especially its active site; the sequence of enzyme-substrate intermediates that occur along the reaction pathway, their individual structures, and the energetic relationships between them. Clearly no single technique can provide information at the level required on all these topics, and a synthesis of data from several disciplines is necessary before correct answers in a very difficult field can be expected.

The contribution of X-ray diffraction, as the unchallenged technique for the determination of three-dimensional structure, would seem to lie in principle in defining the structures of the native enzyme (with essential aid from sequence studies) and the enzyme-substrate intermediates. But, despite great success with the enzymes themselves, structures of true, productive intermediates have proved elusive. The reason for this is that at room (or physiological) temperatures there is such a gross inequality between the rapidity of the enzyme reaction even in the crystalline state, and the length of time required to complete an X-ray experiment, that there is no possibility of success. The only productive intermediates that can possibly be examined in normal conditions are the Michaelis complexes of certain enzymes whose reactions and equilibrium constants are favourable.

The inability to look at true enzyme-substrate intermediates has not prevented X-ray diffraction from producing some important results on the activity of certain enzymes, for example, lysozyme, the serine proteinases and carboxypeptidase. With these enzymes, results from the study of the interactions of inhibitors or virtual substrates could be extended by structural and chemical considerations to

produce plausible models of the productive complexes, from which generally accepted hypotheses of their hydrolytic activity have been deduced. However, the intracellular metabolic enzymes that are now under study have so far resisted this approach, presumably because their more sophisticated mechanisms are not so amenable to chemical intuition. In this context the two papers in the current issue of *Nature* (Fink and Ahmed, page 294 and Alber, Petsko and Tsernoglou, page 297) on the formation and X-ray analysis of stable, productive enzyme-substrate intermediates at very low temperatures, represent a radical departure of great promise.

It has long been known that many enzymes are catalytically active at sub-zero temperatures. Douzou, in his pioneering studies of enzymes at low temperatures (Douzou *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **66**, 787; 1970), has shown that true enzyme-substrate intermediates can be isolated in a stable form at very low temperatures if sufficient care is taken with the physicochemical properties of the aqueous-organic solvent systems that are necessary. In view of this Fink and Ahmed have investigated the behaviour of chymotrypsin, trypsin and elastase towards specific ester substrates at temperatures between -20 and -70 °C in aqueous-organic solvents, to test the feasibility of obtaining stable crystalline intermediates of these enzymes suitable for X-ray studies. They show that in these conditions the rate of acylation is several orders of magnitude faster than the rate of deacylation, and that for each enzyme, conditions can be achieved in which the rate of deacylation becomes so low that acyl-enzyme intermediates accumulate to high concentrations. At temperatures of -50 to -70 °C in aqueous dimethyl sulphoxide or methanol at pH values of maximum activity, they have prepared well-defined acyl-enzyme intermediates in almost stoichiometric quantities, for all three enzymes both in the dissolved and crystalline states. These inter-

mediates are stable for periods of days to months at low temperatures, but when warmed up to 0 °C they are turned over at the expected rate.

A preliminary X-ray analysis of one of the stable acyl-elastase intermediates characterised by Fink and Ahmed is reported by Alber, Petsko and Tsernoglou. Elastase was chosen because its active site is accessible, and it exhibits low solubility and high stability in the aqueous methanol which is necessary for the X-ray studies because its low viscosity allows the substrate to diffuse easily into the crystal. Using a flow-cell they were able to change the mother liquor from 0.01 M sodium acetate to aqueous methanol in a number of stages of increasing concentration and lower temperature until 70% methanol was reached at -55 °C. In these conditions the substrate, N-carbobenzoxy-alanyl-p-nitrophenol ester could be added to the cell and its binding followed crystallographically. After equilibrium was attained three-dimensional X-ray data were collected to 3.5 Å resolution. As the original structure determination (Watson, *et al.*, *Nature*, **225**, 806; 1970) was of the tosyl enzyme, Alber, Petsko and Tsernoglou independently determined the structure of the native enzyme at room temperature, and also collected native data at -55 °C. This enabled them to be clear that the enzyme's structure was essentially undisturbed by the transfer from an ionic liquid at room temperature to an organic liquid at low temperature. The difference map calculated from the data sets collected at -55 °C, although not analysed in any detail in accord with the modest resolution limit, was nevertheless fully consistent with the expected productive acyl-enzyme intermediate in the catalytic process as predicted by Fink and Ahmed. The control experiment of raising the temperature of the crystal to -10 °C caused the binding curve to reverse, and the corresponding difference map showed no density remaining in the active site.

It seems certain that this technique will in due course be used to produce

detailed structural information on the acyl-enzyme intermediate, and probably also on other intermediates, of the serine proteinases. This would come close to realising the full potential of X-ray diffraction studies of enzymes, that of being able to obtain a series of

"stills" of the enzyme-substrate complex at each point of stability along the reaction pathway, which would indeed be a major contribution to the understanding of enzyme activity, however this understanding may be defined. □

Cytoplasmic control of protein synthesis

from Pamela Hamlyn

An EMBO workshop on Cytoplasmic Control of Eukaryotic Protein Synthesis was held on July 19-22, 1976 at King's College, Cambridge and was organised by Tim Hunt and Richard Jackson, Department of Biochemistry, University of Cambridge, and Alan E. Smith, Imperial Cancer Research Fund, London.

For many workers in the field of eukaryotic protein synthesis, control of initiation of polypeptide formation is synonymous with translational control. A suitable starting point for the conference was, therefore, to consider the protein initiation factors which regulate the various steps during initiation. Two groups have purified to homogeneity the initiation factors from reticulocytes. W. C. Merrick described the work of the National Institutes of Health group and T. Staehlin that of the Basel Institute for Immunology. Each group has its own system of naming the different initiation factors, but the physical and functional characterisation of the proteins is sufficient to allow them to make correlations with confidence. Four initiation proteins have been isolated from Krebs II ascites cells. H. H. Dahl (ICRF, London) explained their functional activities and described experiments in which reticulocyte factors had been successfully substituted for ascites factors. In the past some initiation factors were claimed to be messenger-specific. Most workers now think that such observations were due to inadequacies in the assay systems used and agree that initiation factors are functionally homologous in different tissues and species.

Tails and caps

Messenger RNAs may have poly(A) tails, 'caps' (blocked and methylated 5' termini) and untranslated sequences either side of the amino acid specifying region. Since these structures may be involved in translational control mechanisms, part of the conference was devoted to them. G. Marbaix (University of Brussels) confirmed that the poly(A) tail confers stability on globin

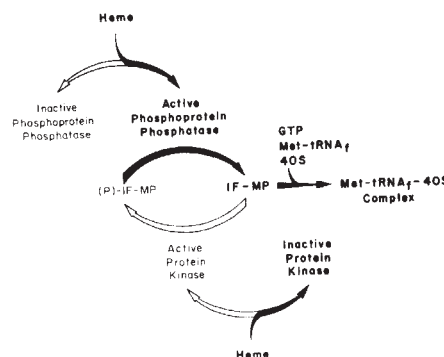
mRNA injected into oocytes and showed that messenger lacking poly(A) was only degraded once its translation had been initiated. R. Kaempfer (Hebrew University, Jerusalem) showed that globin mRNA requires the 3' non-coding region for its translation but not the poly(A) tail.

The work on the cap region is in an earlier stage, at which many experimental results seem contradictory. In his summary of this group of lectures the chairman, B. P. Perry (Institute for Cancer Research, Philadelphia), concluded that the effects of cap on translation were quantitative and seemed to depend on the messenger, the cell-free system used and the conditions of translation.

Inhibition

The section on the control of initiation centred on the nature of the inhibitor of protein synthesis formed when a reticulocyte lysate is incubated without the addition of haemin. Isolation of the inhibitor results in the copurification of a phosphorylase activity which can phosphorylate the initiation factor responsible for the formation of the ternary complex between GTP and met-tRNA_f. The phosphorylated factor is inactive. Does the phosphorylation cause the inactivation, or is it in some way coincidental? The group from the University of Cambridge have isolated an inhibitor which specifically phosphorylates *de novo* the small subunit of initiation factor IF-E2 (IF-MP). The copurifying kinase and inhibitory activity are heat inactivated at the same rate. Further evidence that phosphorylation of IF-E2 is the inhibitory event is provided by experiments of B. Hardesty (University of Texas) whose group has shown that antibodies to the inhibitory factor, which prevent it acting as an inhibitor, also prevent the phosphorylation of IF-E2. The group from MIT have broadly similar results but are more cautious in their interpretation, pointing out that phosphorylation of IF-E2 may be a normal event at some stage in protein synthesis and that the activation of the inhibitor may alter the normal phosphorylation pattern of IF-E2 and somehow prevent the recycling of the factor. The work

of M. J. Clemens (National Institute for Medical Research, London) indicates that phosphorylation alone is not sufficient to inhibit binding of IF-E2 to the GTP-Met-tRNA_f complex. J. A. Traugh (University of California) described phosphorylase activities isolated from a haemin-stimulated reticulocyte lysate. The diagram below illustrates a working model of how she thinks the removal and addition of phosphate to the initiation factor might be involved in the control of polypeptide initiation.



Discussing the action of double-stranded RNA in preventing the translation of encephalomyocarditis virus RNA in an L cell lysate containing interferon, I. M. Kerr (NIMR, London) concluded that there are two steps in the inhibition of translation and hinted that a protein kinase(s) may have a regulatory role in the activation of the inhibitor. In his introduction to the section on interferon and protein synthesis, P. Lengyel (Yale University) pointed out that the effects of interferon were probably many and various. This was borne out by the speakers in this section: no consistent picture of its action emerged.

Developing systems

Some of the best known examples of cytoplasmic control of protein synthesis have been observed in developing systems. mRNA is stored in the cytoplasm and not translated until a further stage in development. Most of the speakers have approached this problem by studying the mRNA before and during its translation to see if there were detectable differences in its properties. C. H. Darnbrough (University of Edinburgh) concluded that, of two populations of messengers with large and small poly(A) size, the latter increased and the former decreased during oogenesis in *Xenopus*. Comparison of the 'active' mRNA isolated from polysomes and the stored RNA from ribonucleoprotein particles, led M. E. Buckingham (Pasteur Institute) to propose that in dividing muscle cells poly(A) tails are involved in maintaining mRNA conformation, rather than

RESULTS obtained by Melton and Giardini (page 309 of this issue of *Nature*) suggesting that oxygen not nitrogen might be the dominant impurity in diamond pose a problem in the light of previous work. In 1936, Robertson *et al.* (*Proc. R. Soc. Lond.*, **A157**, 579–593) classified diamonds into two types by their optical absorption spectra. Kaiser and Bond (*Phys. Rev.*, **115**, 857–863; 1959) using mass spectroscopy, showed that nitrogen impurity was the microscopic basis of this classification; type I diamonds contained nitrogen, type II did not. The nitrogen concentration correlated with the optical absorption lines noted by Robertson *et al.* Lightowers and Dean (*Diamond Res.*, 21–25; 1964), using activation analysis, confirmed Kaiser and Bond's results. In some diamonds the nitrogen is paramagnetic, and can be detected unambiguously by spin resonance. Thus there is no doubt that nitrogen is a major impurity in diamond—up to 0.3%—a massive concentration for an impurity in a crystal.

Kaiser and Bond also found small amounts of hydrogen and oxygen. Oxygen has been confirmed by Sellschop (*Diamond Res.*, 35–41; 1975) using activation analysis.

One of the problems in determining the impurity content of diamond is that there are often trapped inclusions of other minerals. These are important, particularly to the geologist, because they indicate the growth environment of the diamond; but they may give a misleading picture of which elements can genuinely enter the diamond lattice as substitutional or interstitial impurities. Kaiser and Bond, and Lightowers and Dean used stones without visible inclusions. Even so, Sellschop has shown that all diamonds contain submicroscopic inclusions, and these

account for most of the oxygen impurity.

Using mass spectrometry, Melton and Giardini (*Amer. Mineral.*, **59**, 775–782; 1974 and **60**, 413–417; 1975) found that some macroscopic inclusions contain gases—hydrogen, methane, nitrogen, carbon monoxide and water.

Impurities in diamonds

from John Walker

The most abundant element was hydrogen, followed by oxygen and nitrogen. The results are not in conflict with previous studies, because hydrogen cannot be detected by activation analysis, and nitrogen and oxygen need special techniques. The most comparable work is that of Kaiser and Bond, but even here there is no conflict. The latter selected "inclusion-free" diamonds, whereas Melton and Giardini chose crystals containing inclusions, and studied the inclusions.

Thus the picture of impurities in diamond was complicated but consistent. The geologist studies the inclusions; the physicist, the genuine diamond lattice. In particular the physicist, whether he is concerned with optical, electrical, mechanical or thermal properties, knows that type I and type II diamonds have quite different properties, because of their differing nitrogen content.

This consistent picture has been upset by Melton and Giardini's latest paper. They used "inclusion-free" diamonds, and instead of crushing them as previously, they graphitised them at 2,000 °C like Kaiser and Bond. The results are surprising. "The gases released from the diamond, in decreasing

order of abundance, were CO, H₂, H₂O, CO₂, N₂, CH₄, and Ar. The atomic weight percent is O=59.2%, C=28.1%, H=10%, N=2.5% and Ar=0.2%. These data are consistent with the gases released by the crushing of numerous other diamonds *in vacuo*."

Since nitrogen is currently thought to be the dominant impurity in diamond, how can we explain this discrepancy? Melton and Giardini suggest nitrogen contamination from the carbon crucible used by Kaiser and Bond. But this is unlikely because Kaiser and Bond's results are internally consistent—the nitrogen concentration correlated with optical absorption in their crystals. And Lightowers and Dean, using a different technique, got the same result.

A possible explanation is that the diamonds came from different sources. This is known to affect impurity content. In any case, the fact that Melton and Giardini's diamonds are oxygen-rich, surprising though it may be, does not contradict Kaiser and Bond's results on nitrogen. Unfortunately, Melton and Giardini do not give the concentrations of oxygen and nitrogen they found. My estimates based on their data suggest that both lie within the range of accepted values; but it would be better to have the authors' own figures.

One hopes that Melton and Giardini will extend their graphitisation work to diamonds from other sources, and measure the optical spectra before graphitisation, to check Kaiser and Bond's correlations. If the same diamonds could also be studied beforehand by the non-destructive technique of activation analysis, to compare results, the puzzle would probably be resolved. Diamond researchers will await further results with interest.

conferring differential stability. T. Humphreys (University of Hawaii) reported that on fertilisation of sea urchin eggs messenger translation is increased—the messengers are stable but their poly(A) turns over rapidly. Using the same system, G. Giudice (Institute of Comparative Anatomy, Palermo) has shown that although capping does not cause the increase in translational efficiency it may be involved in mRNA selection.

R. A. Laskey (MRC Laboratory of Molecular Biology, Cambridge) has examined the effect on endogenous protein synthesis of injecting polysomes and mRNA into *X. laevis* oocytes. He maintains that oocytes do not have 'extra translational capacity' and that by choosing conditions that accurately reflect the amount of protein synthesised the endogenous protein synthesis decreases competitively with added exogenous mRNA. There is no

competition when the messenger is injected with its own ribosomes. Laskey concludes that the amount of protein synthesised is regulated by a component of normal polysomes and not by messenger availability.

Virus infection

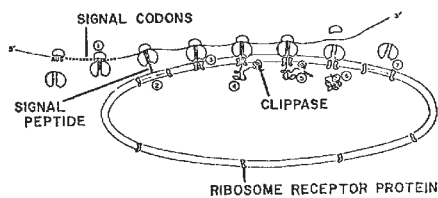
The series of lectures on the control of translation during viral infection was similar to those on masked messengers in development in that many interesting systems were described without providing much insight into the mechanism of control. In general, host mRNA translation is suppressed at the expense of viral protein production. The herpes simplex system was described by C. M. Preston (MRC Virology Unit, Glasgow). He has shown that the effect of this virus on tissue culture cells can be reproduced in a lysate of the cells. The lysate can also respond to exogenous mRNA, but its response is decreased

if the lysate is prepared from infected cells. The lesion can be traced to the ribosomes of the infected cells, since proteins washed off reticulocyte ribosomes by salt treatment restore the translational capacity. R. E. Thach (Washington University) has shown that EMC RNA can 'out-compete' host mRNAs but that the competition is relieved by an excess of the initiation factor corresponding to rabbit IF-E6 (IF-M3). L. Carrasco (ICRF, London) has found that after infection with picornavirus, changes take place in the cell membrane which impair the sodium transport system, leading to an increase in the cell concentration of sodium ions and a decrease in potassium. Experimental evidence has confirmed his suggestion that increased sodium concentration favours the translation of viral, rather than host, mRNAs. It may be that at these altered salt conditions binding of initia-

tion factors to viral messengers is favoured—hence their preferential translation.

In the section dealing with the topography of protein synthesis G. Blobel (Rockefeller University, New York) presented a very appealing model to explain the translation of mRNA for secretory proteins on membrane-bound ribosomes. It requires that mRNAs for secretory proteins code for an N-terminal peptide that is recognised by the membrane, resulting in the attachment of ribosomes to the membrane so that the growing protein is vectorially transported into the intracisternal space. This is illustrated in the diagram: the numbers indicate sequential steps in the synthesis of the protein. Bernard Mechler (University of Cambridge) proposes a slightly different scheme since he has to explain his observation that the binding of ribosomes to the membrane can be mediated by the mRNA directly

rather than its translational product. H. Lodish (MIT, Cambridge) showed that translation of the polycistronic Sindbis virus RNA can take place in the cytoplasm or on membranes using the same ribosomes but producing different proteins. He concludes that the N-terminus of the nascent chain



must specify the binding of ribosomes to membranes.

The participants left the conference with the impression of many examples of cytoplasmic control of protein synthesis but few explanations of the mechanisms involved, an admirable situation sure to keep them in business for several years to come. □

diminished in a series of steps as the flow velocity was increased.

The most definite and interesting observation, however, was the remarkably small velocity at which the signal disappeared. The exact value depended on temperature, but fell within the range $0.003\text{--}0.02\text{ mm s}^{-1}$. These velocities are several orders of magnitude smaller than the Landau critical velocity at which the superfluidity would be expected to disappear as a result of pair-breaking, so that some other mechanism must presumably be involved. It is interesting that investigations of the heat conduction by R. T. Johnson, R. L. Kleinberg, R. A. Webb and J. C. Wheatley (*J. Low Temp. Phys.*, **18**, 501; 1975) also yielded multiple critical velocities for the superfluid which were remarkably small ($0.2\text{--}0.8\text{ mm s}^{-1}$), although considerably larger than the velocities reported here. The authors demonstrate, however, that the two sets of measurements may in fact be in quantitative agreement if it is assumed that the flow did not occur through a transfer of bulk liquid: if only the superfluid component of the liquid had moved, leaving the normal fluid component clamped in the tube by its own viscosity, it would have had to move relatively briskly to simulate a given rate of apparent bulk flow because it constitutes only a small proportion of the liquid at the temperatures in question. On this basis they deduce superflow critical velocities in the range $0.14\text{--}0.64\text{ mm s}^{-1}$, in good agreement with those derived from the thermal conduction experiments.

Why should these critical velocities be so small? One is immediately reminded of the parallel situation in superfluid ^4He , where the actual critical velocities for superflow are usually about 1 mm s^{-1} , compared with the much larger Landau critical velocity for roton creation of around 50 m s^{-1} . In the case of ^4He , the discrepancy has been shown to arise from the relative ease with which quantised vortex lines may be generated in the liquid for flow velocities far below the Landau velocity. It seems very likely that something of a similar nature is happening in ^3He . The situation is, of course, infinitely more complicated because the liquid is governed by a vector order parameter, is anisotropic, and takes up complicated textures depending on the shape of the container, applied electric and magnetic fields, and so on. Furthermore, it is not yet by any means clear what would be the analogue of a vortex, in the case of ^3He : there may in fact be several different sorts of collective excitation of this general nature.

Assuming, however, that the low critical velocities observed in these experiments arise from the onset of

Flow of superfluid ^3He

from P. V. E. McClintock

To push some of the newly discovered superfluid ^3He through a fine tube, in order to see how it behaved, might seem a particularly obvious experiment, were it not for the daunting difficulties inherent in working at temperatures near 2 mK . In fact, it is a quite remarkable achievement in terms of experimental design and technique that has enabled R. M. Mueller, E. B. Flint and E. D. Adams to report (*Phys. Rev. Lett.*, **36**, 1460; 1976) the first studies of equilibrium flow phenomena, only four years after observation of the superfluid phases was first suspected. Their experiments were carried out in the Physics Department at the University of Florida.

Because of extreme difficulties of making thermal contact in this ultra-low temperature range, it is usually necessary to design the cryogenics and the experimental cell as an integral unit. Accordingly, the Florida group have devised an ingenious arrangement whereby a pair of Pomeranchuk cells was used both for cooling the ^3He into the superfluid A-phase and also for inducing a flow of liquid through the narrow tube which connected them together.

The Pomeranchuk cooling technique depends on the fact that, at very low temperatures, ^3He gets colder if it is solidified by compression. Thus, by applying a mechanical force to reduce the volume of a sample of ^3He enclosed in a flexible chamber, thereby solidifying some of it, the remaining liquid may be cooled through the superfluid

transition at 2.6 mK . The mechanical force is usually generated by way of a separate hydraulic system in which the working fluid is liquid ^4He .

The two Pomeranchuk cells of the Florida apparatus were fitted with completely separate hydraulic systems, so that their volumes could be varied independently of each other. By compressing both cells simultaneously the ^3He could be cooled without inducing any movement of liquid in the connecting tube. On the other hand, if one cell was compressed while the other was being expanded, then liquid could be made to flow through the tube at constant temperature. A nuclear magnetic resonance (NMR) technique was used for monitoring events in the flow tube.

In view of a calculation by Fetter (*Phys. Lett.*, **54A**, 63; 1975), it had been anticipated that the flow would result in a shift downwards of the NMR resonant frequency. It was surprising, therefore, that rather than changing its frequency, the resonance line in practice developed satellites, and then abruptly disappeared, as the flow velocity was increased. Although the behaviour was not always reproducible in detail, it was found that the satellite structure never appeared when the external magnetic field was applied at 90° to the flow direction. This may perhaps be related to Fetter's prediction that no frequency shift should be seen in this configuration. A further complication was that, before its final rapid fall, the resonance signal sometimes

dissipative processes, it seems probable that at least one completely new type of collective excitation is being created, thus promising to add yet another whole dimension of complexity to our understanding of this extraordinarily complicated liquid. □

Two approaches to gene synthesis

from Maria Szekely

THE recent discovery that synthetic genes can be used to produce genetic recombinants (see *News and Views*, 260, 189; 1976) opens wide possibilities in the field of genetic engineering. Well-characterised DNA sequences can be inserted into plasmid DNA thus providing specific probes with known sequences for studying the organisation and expression of eukaryotic genomes. Amplification of the inserted gene yields large amounts of specific sequences which can be used for the production or isolation of highly labelled mRNAs, for sequence studies, and so on. At the same time, the safety risk is lessened, as no unknown, harmful genetic information can be integrated into the recombinant.

So far only one eukaryotic gene, that of β -globin, has been inserted into bacterial plasmids. Almost simultaneously, four laboratories reported the production of recombinants from β -globin DNA, using different techniques but all of them starting with reverse transcription of globin mRNA into cDNA. (Rougeon *et al.*, *Nucleic Acids Res.*, 2, 2365, 1975; Efstratiadis *et al.*, *Cell*, 7, 279, 1976; Rabbits, *Nature*, 260, 221, 1976; Higuchi *et al.*, *Abstract ICN-UCLA Winter Conference*, 1976). The advantages of this technique are obvious, there are, however, also some limitations to the method. It follows from the principle of the technique that the DNA inserted into the plasmid can contain only the sequences present in the mRNA: any additional, non-transcribed sequences that may be present in the original gene will be lost. It remains to be seen how their absence affects the function of the integrated gene. In practice, the limitations go even a step further: it depends on the efficiency of the reverse transcriptase how much of the genetic information present in the mRNA will be passed on to the synthetic gene. In many laboratories only fragments of cDNA copies could be obtained with this enzyme. Rougeon *et al.* used a 300–400 nucleotide long cDNA preparation to synthesise the double-stranded globin DNA and after amplification ended up with a 100 to 150

base-pair long integrated globin DNA, about one fifth of the length of the mRNA.

Maniatis' group reported last year the synthesis of a full-length copy of globin mRNA (Efstratiadis *et al.*, *Cell*, 4, 367; 1975). A few months ago the same group described the synthesis of double-stranded globin DNA with the aid of DNA polymerase I, making use of the hairpin loop structure at the 3'-end of this cDNA, which served as primer in the synthesis of the anti-cDNA strand. (Efstratiadis *et al.*, *Cell*, 7, 279; 1976). They succeeded in inserting this synthetic gene into PMB 9 plasmid DNA and in their recent paper (Maniatis, Kee, Efstratiadis and Kafatos, *Cell*, 8, 163; 1976) they describe in detail the techniques of integration of the DNA, transformation of *Escherichia coli* cells and identification of the recombinant.

The synthetic gene is 580 base pairs long, about 80 nucleotides shorter than β -globin mRNA. It contains the full coding region plus 40 and 110 nucleotides of the untranslated regions at the 5' and 3' termini, respectively. Molecular hybrids were constructed by annealing the globin DNA which carried poly(dT) tails to *Eco*RI-treated plasmid DNA to which poly(dA) tails have been attached. Transformation of *E. coli* HB101 with this hybrid DNA yielded 600 tetracycline-resistant colonies, a high proportion of which contained sequences of the β -globin gene, as was shown in hybridisation assays. The size of the inserted DNA did not change upon construction of the molecular hybrid, transformation and cloning.

The method seems suitable for general application in the synthesis and amplification of eukaryotic genes. Maniatis' group has already synthesised a number of other genes by copying eukaryotic mRNAs.

It is essential for the usefulness of such techniques that the original structure of the gene should be retained in the recombinant. Fidelity in copying the mRNA and stability of the DNA structure during integration and transformation is therefore of vital importance. Maniatis *et al.* characterised the synthetic gene and the inserted globin DNA after amplification by comparing restriction maps of the DNAs and correlating them to the structure of globin mRNA. Using eight restriction enzymes with known recognition sequences, the restriction map of synthetic DNA was established and compared with the cleavage pattern predicted from the nucleotide sequence of β -globin mRNA. As only part of the nucleotide sequence has been determined so far, some cleavage sites were established by deducing the nucleotide sequence from the known amino acid sequence of β -

globin. The restriction map of synthetic globin DNA was in perfect agreement with that expected from the mRNA structure.

The same restriction enzymes were used individually and in combination on the recombinant DNA. By using *Hha* endonuclease, an enzyme which does not cleave synthetic globin DNA but produces many fragments from PMB 9 DNA, one fragment, carrying the inserted globin gene could be isolated and characterised. The restriction map of this fragment was established, and the orientation of the inserted DNA and the distance between the restriction sites determined. These proved to be the same as in the synthetic gene before integration, confirming that the entire synthetic globin DNA molecule was amplified without sequence rearrangements. Some slight changes were detected in the poly(dA)–poly(dT) tails only.

The approach used by Maniatis' group yields very useful DNA probes which will greatly enhance progress in our understanding of the structure and function of the eukaryotic genome. The success of a different, more chemical approach has been reported recently from Khorana's laboratory: the complete gene of suppressor tyrosine tRNA of *E. coli* has been synthesised without using an RNA template. The techniques were worked out years ago by Khorana's group. Following a known nucleotide sequence, chemical synthesis is used to produce blocks of nucleotides and the ligase reaction to join them. The synthetic gene contains the total sequence of the precursor tRNA and includes the sequence of the promoter site and of the stop signal for transcription. Twenty-four scientists contributed to its synthesis which has taken 9 years. This gene has now been introduced into an *E. coli* amber mutant by way of a transducing phage (the details of the technique have not been published so far) and it was found to function correctly as a suppressor gene in the bacterial cell.

Khorana's method requires more time and work than the copying of an RNA template and its use is limited to the synthesis of genes the sequence of which has been fully established. Still, this approach has some great advantages over the reverse transcription of RNAs. The gene synthesised by Khorana contains also sequences which are not present in the final gene product, sequences lost during processing of the RNA and non-transcribed control signals. Such sequences cannot be incorporated into a reverse transcript. The purely synthetic method also makes it possible to introduce specific alterations into the gene structure and to study the effect of such "mutations". It would be interesting to

see if the two approaches could be combined and a new technique developed which would unite the relative simplicity of the copying technique with the further possibilities provided by the methods of chemical synthesis. □

New method for measuring nuclear lifetimes

from P. E. Hodgson

AN ingenious method for measuring very short nuclear lifetimes has recently been developed that greatly extends the range covered by existing techniques. This is an important advance because these lifetimes can often be calculated from nuclear wavefunctions, and thus provide a sensitive test of the theories used to calculate the wavefunctions.

Nuclear lifetimes are usually measured by the delayed coincidence and the Doppler shift techniques, and these cover a wide range of times greater than about 10^{-15} s. For shorter lifetimes there has hitherto been only the blocking technique which is restricted to a few suitable materials. The new method seems likely to provide a way of measuring lifetimes from about 2×10^{-15} to 6×10^{-18} s, and thus extends the region of measurable lifetimes by more than two orders of magnitude.

The new method is based on a very simple idea. If a nucleus of charge Z decays by electron capture to an excited state of another nucleus of charge $(Z-1)$ a vacancy is left in the atomic K-shell from which the electron has been captured. This vacancy will soon be filled by a transition from a higher atomic state with the emission

of an X ray. Now if the X-ray transition takes place before the nuclear state decays, its frequency is characteristic of the nucleus of charge $(Z-1)$. But if the nucleus decays by proton emission before the X-ray transition takes place, then the X ray will have a frequency characteristic of the nucleus of charge $(Z-2)$. Since these X-ray frequencies can easily be measured, it is easy to find out whether the nuclear decay took place before or after the atomic decay. In practice there is of course a distribution of decay times for both processes, so it is the relative proportion of the two types of decays that is measured and this gives the ratio of the nuclear and atomic lifetimes. Since the atomic lifetimes are well known both experimentally and theoretically, the nuclear lifetime is found.

This method has been devised by a group of scientists from the Chalk River Laboratories and the University of Toronto in Canada. They have obtained data on the lifetimes of ^{69}Ga , ^{69}As , ^{73}Br , and ^{77}Rb , and in their first publication (*Phys. Rev. Lett.*, **37**, 133; 1976) they give details of their result for ^{69}As .

In this case the decay sequence starts with $^{69}_{34}\text{Se}$ undergoing electron capture to an excited state of $^{69}_{33}\text{As}$, which in turn undergoes proton emission to a state of $^{68}_{32}\text{Ge}$.

The initial nucleus ^{69}Se was produced in the $^{40}\text{Ca}(^{32}\text{S}, 2\text{pn})^{69}\text{Se}$ reaction using the 100 MeV beam of the Chalk River tandem Van de Graaff accelerator. After irradiation, the target was moved rapidly to a counting position where arrays of counters measured the X rays from the atomic transitions, the delayed protons from the decay of the ^{69}As to ^{68}Ge , and the gamma rays from the nuclear transitions in As.

The histogram in the figure shows the energy spectrum of all the X rays in coincidence with the delayed protons, compared with normalised curves corresponding to the decays of Ge and As found in independent measurements. It is clear that the histogram can be very well fitted by the sum of these two curves.

Many states of ^{69}As can be reached by electron capture, so the decay protons are themselves varying in energy. Since the relation between the relative numbers of Ge and As X rays depends on the proton energy, so do the lifetimes of the states of As. The lifetimes of the states in As vary from 2×10^{-16} to 10×10^{-16} s. These numbers are average lifetimes for successive bands of states and rather detailed calculations based on the statistical theory of nuclei are required to interpret them in detail.

If there had been only one state in As fed by the electron capture in Se the experiment would have given an

unambiguous determination of its lifetime. The technique is clearly successful and is likely to be applied to determine the lifetimes of many states, and hence to increase our knowledge of nuclear structure. □

Effects of a long hot summer

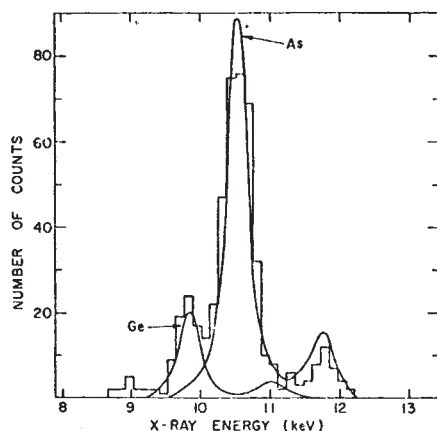
from Peter D. Moore

THE unusually warm and dry summer experienced in Britain this year has undoubtedly influenced our vegetative cover. Apart from widespread damage by fire, the high temperatures and low water availability have themselves produced some very evident changes. Roadside verges and lawns are parched, with only the deep-rooted rosette species showing signs of life. Among trees, birch appears particularly susceptible and dead birches are now abundant on heathlands and in mixed woodland both in the lowland and the upland zones of the country.

Many of the common species affected will probably recover quickly and even if they do not, any change in status will be difficult, if not impossible, to assess. There are certain species, however, which may merit especial attention at this time, such as those which lie at their climatic limit in Britain and which may expand or contract their range as a result of the effects of this and preceeding summers. There are also those species which are restricted to lower altitudes in the British Isles because of the shorter growing season in higher regions; they may attain new heights. And there are those arctic alpine species which are limited by high summer temperatures and which may, therefore, be suffering.

One species which reaches its north-western limit of distribution in the British Isles and which might be expected to respond to the present conditions is the stemless thistle (*Cirsium acaulon*). Pigott (in *The Flora of a Changing Britain*, edit. by Perring, F., 32, Classey, Hampton, Middlesex, 1970) has shown that the temperature of the flowering head of this plant is critical in determining the rate of development of the embryo. Only in southern and eastern Britain does the plant normally produce viable seed, though some outlying populations in the north and west appear to have been established during exceptionally warm summers.

Many plants are common at fairly low altitudes and yet fail to flourish on mountain summits because of their inability to grow adequately, or flower, or produce mature, viable seed, within



Histogram showing the spectrum of all X rays in coincidence with all decay protons. The curves show the spectra measured independently for the As and Ge decays.

NEW observations of H- β or Lyman- α fluxes of a sample of 57 Seyfert galaxies and QSOs suggest strongly that QSOs are indeed distant, bright Seyfert galaxies too remote for the faint outer structure of the galaxies to be observed. As well as confirming the now increasingly accepted view that the redshifts of QSOs are cosmological distance indicators, the suggestion that QSOs are high-redshift Seyferts provides a new use of these objects as cosmological probes, and suggests some limits on the so-called "deceleration parameter" of our Universe.

The possibility of a link between active galaxies such as Seyferts and QSOs has long been recognised in a qualitative sense, two touted possibilities being that explosions on the scale of a QSO may occur in the nuclei of all spiral galaxies from time to time, or that such violent activity may mark a step in the evolution of a proto-galaxy, after which it settles down into a quiet spiral with no further comparable activity. Supporters of such ideas have had powerful circumstantial evidence to hand in the observation that class I Seyferts and QSOs cannot be distinguished from their spectra alone. Both have broad Balmer emission lines, narrow forbidden lines and power-law spectra, the difference being that Seyferts are seen as active nuclei surrounded by visible galactic material, while QSOs have higher redshifts and no surrounding galaxies are seen. Because of the confusion effects from bright

nuclei, surrounding galactic disks could not, however, be observed at redshift greater than 0.1—so any Seyfert of class I with $z > 0.1$ would be identified automatically as a QSO.

Weedman has now developed from this observation a quantitative

Seyfert galaxies and QSOs

from John Gribbin

measure of the similarity between the two kinds of object (*Astrophys. J.*, **208**, 30–36; 1976). Earlier studies along these lines had compared the fluxes from the two in a specified part of the observed spectrum; as Weedman points out, this is not the best approach because the redshift effect means that different parts of the emitted spectra are shifted into the chosen observational window. Instead, he has made measurements of the emission of his sample of Seyferts and QSOs in the hydrogen lines, H- β or Lyman- α . The H- β lines were measured wherever possible, but at large redshift these are shifted out of the visible range and for those objects L- α can be measured instead, the relative strength of the two lines (based on model calculations by K. Davidson, *Astrophys. J.*, **171**, 213; 1972) then giving an estimate of the strength of the invisible H- β .

The results show clearly a continuous smooth distribution of luminosity in line with the cosmological interpretation of the redshifts of these objects. The range covered, equivalent to 11.5 magnitudes, is large, but as Weedman points out still less than the luminosity range of main sequence stars. Accepting this as confirmation that QSOs are simply bright class I Seyferts, this immediately provides a new probe of the Universe extending over a wide range of redshifts, and Weedman points out some preliminary conclusions from this which set limits on some cosmological parameters. In particular, the luminosities of high redshift QSOs (which are now regarded as high-redshift Seyferts) are relatively faint, suggesting that either there is some limit to the brightness of these objects or that the deceleration parameter of the Universe, q_0 , is negative. This tentative first conclusion is, however, less important than the fact that at last it seems possible that QSOs can be used with confidence as genuine cosmological probes to define the structure of our Universe on the large scale, and to rule out some of the large family of possible models now applied to the Universe. The problem of the energy source of QSOs remains, of course (even if there is some upper limit), but here too it seems that this problem can now be approached from a new and promising direction by investigating the more easily studied and nearer members of the family, the Seyfert galaxies.

a short growing season. Pearsall (*Mountains and Moorlands*, 49, Collins, 1956) described his observations on the heath rush (*Juncus squarrosus*) and showed how the length of the flowering stalk, the number of flowers per inflorescence and the number of viable seeds produced per capsule are all inversely related to altitude. The production of viable seed for this species is uncommon at altitudes over 820 m. It is also reported, however, that the heath rush produced seed on the summit of Ben Wyvis (1,036 m) in the unusually warm summer of 1947.

Among lowland plants, some occur only in those parts of the country experiencing high summer temperatures. For example, the round-headed rampion (*Phyteuma tenerum*) occurs mainly within the 16.6 °C July average isotherm. Such species may also be expected to extend their range under current conditions.

Other species may find high summer temperatures detrimental, particularly species of arctic alpine distribution. Dahl (*Oikos*, **3**, 22; 1951) observed that

arctic alpine species grown in lowland gardens suffered most during hot summers. He has also demonstrated that many of these species have distributional patterns in which their limits coincide with low summer temperatures. Similar observations have been made recently for certain arctic alpine species in Newfoundland (Damman, *Can. J. Bot.*, **54**, 1561; 1976), for example, the bog bilberry (*Vaccinium uliginosum*) is found only in areas having an average temperature in the growing season below 15 °C. It is known that dark respiration rates of many arctic alpine species are higher than their lowland counterparts (Billings and Mooney, *Biol. Rev.*, **43**, 481; 1968) and since respiration is stimulated by high temperatures, the plant could experience difficulty in maintaining a positive carbon balance during a hot summer.

One species which does appear to be suffering in upland Britain this year is the bilberry (*Vaccinium myrtillus*). This is a species which dominates some open moorlands in the uplands of western Britain, yet is found only under a

woodland canopy in the east and in continental Europe. Microclimatic factors, possibly involving temperature and humidity may therefore be critical for its survival. This summer the open populations of the west are showing evident signs of stress when unprotected by a tree cover. It is possible that its reduced vigour will allow such species as the heather (*Calluna vulgaris*) to spread at its expense.

Our freak summer offers valuable opportunities to increase our understanding of many plant species and one can only hope that any ensuing changes in vegetation will not remain unrecorded. This may be an appropriate time to mobilise that large and willing labour force, the amateur botanists, to undertake a census of selected plant species on the model of the British Trust for Ornithology's common bird census. Perhaps the Botanical Society of the British Isles is the appropriate organisation to initiate such a census which could begin to provide an answer to the question how responsive and fluid is our flora? □

Structure and function of haemocyanin

from J. V. Bannister and H. A. O. Hill

The V Haemocyanin Workshop sponsored by the European Molecular Biology Organisation was held on August 1-4, 1976, at the new Marine Biology Laboratory of the University of Malta. The meeting was organised by the Department of Physiology and Biochemistry of the University.

THE haemocyanins are multisubunit copper proteins with respiratory function found in arthropods and molluscs. The molecules are of giant size with a molecular weight of up to 1 million in arthropods, 4.5 million in cephalopods and 9 million in gastropods. The stoichiometry of oxygen binding is one O_2 per two Cu and the copper content indicates a minimum functional subunit of 75,000 in arthropod and 50,000 in molluscan haemocyanin. Subunits of 75,000 molecular weight appear to exist in arthropod haemocyanin and can be regarded as monomers. The monomer molecular weight in molluscan haemocyanin has not been fully resolved.

The study of the structure and behaviour of these extra-large molecules is a matter of real importance, providing as it does a stepping stone to the understanding of the regulation and assembly of still larger biological structures, such as microtubules or ribosomes (J. Wyman, University of Rome). Three-dimensional image reconstruction from electron micrographs has provided a model for gastropod haemocyanins (Mellema and Klug, *Nature*, **239**, 146; 1972) which showed the molecule to consist of a hollow cylindrical drum closed at both ends with material of fivefold symmetry ("collar") containing a "cap" in the centre. The wall has approximately tenfold rotational symmetry and consists of 60 quasi-equivalent morphological units bounded by two dominant sets of helical grooves. E. F. J. Van Bruggen (University of Groningen) presented results on the possible folding of a long polypeptide chain in the strongly curved wall segments from a half molecule. A segment would accommodate one-twentieth of the molecule of *Helix pomatia* haemocyanin and one-fifth of the fivefold "collar". The "cap" of Mellema and Klug is thought to be an artefact. It has proved difficult to dissociate gastropod haemocyanins into material smaller than one-twentieth of the molecule (about 450,000 molecular

weight) without breakage of peptide bonds. The twentieths have therefore been regarded as the monomers of these haemocyanins. If so a monomer contains about nine oxygen binding sites or "domains". C. Gielens (University of Leuven) showed that two sites between the domains are susceptible to proteolysis. Various proteolytic enzymes including subtilisin, papain and Pronase give 50,000 molecular weight fragments with which, *Murex trunculus* haemocyanin (J. Bannister, University of Malta). Similar fragments were observed after prolonged reduction followed by carboxymethylation of the haemocyanin. The protomer may therefore be as small as a single O_2 binding domain in this haemocyanin.

The structure of arthropod haemocyanins seem less perplexing. The assembly of 60S molecules in *Limulus*, and 34S in scorpion and tarantula haemocyanins, from 16S subunits was discussed (E. F. J. Van Bruggen, University of Groningen; J. Lamy, University of Tours; B. Linzen and R. Loewe, University of Munich). Each 16S particle contains six monomers (5S). B. Salvato (University of Padova) questioned this structure of the 16S subunit in *Limulus* haemocyanin. W. Love (John Hopkins University) reported the conditions in which whole molecules of *Limulus* haemocyanin and some of its subunits can be crystallised. The crystal forms from which preliminary X-ray data were obtained are still unsuitable for high resolution structure analysis and conditions are being sought for the growth of more suitable crystals. The 60S whole molecule is predicted by X-ray diffraction to consist of about 50 subunits of which there are at least nine different kinds present in unknown ratio.

Limulus haemocyanin can be dissociated into five distinct subunits (I-V) of about 66,000 molecular weight. The fully associated molecule is therefore a 48-meric structure. Van Bruggen discussed the association of subunits I-V as seen in electron micrographs. The 5S structure is a roughly spherical protein particle with a diameter of about 65 Å. None of the separated subunit fractions was found to be capable of forming an intact 60S structure by itself. The 60S structure is formed by the stacking of two-bridged tetramers probably in a staggered arrangement. The heterogeneity of the 5S subunits was demonstrated in scorpion haemocyanin by

Lamy. Six subunits could be isolated from the haemocyanin, one of which—subunit 4—was shown to have a particular ability to polymerise in one, probably hexameric 16S particle.

Until now the O_2 bound in haemocyanin has been considered to form a peroxo complex with two Cu(II) atoms. R. Lontic (Leuven) proposed consideration of a Cu(I) . . . Cu(III) complex. X-ray photoelectron spectroscopy, however, indicates that Cu in oxyhaemocyanin is cupric because the $2p_{3/2}$ and $3p_{3/2}$ binding energies of the Cu are similar to those in Cu-Zn superoxide dismutase (H. Van der Deen, Groningen).

Oxyhaemocyanins have a strong absorption band in the near ultraviolet. Photo-oxidation experiments with light at the absorption wavelength show that tryptophan is located near the copper-oxygen site (G. Jori, Padova). M. De Ley (Leuven) proposed that photo-oxidation of haemocyanin results in the activation of oxygen by tryptophan.

Haemocyanins show both a reverse and a normal Bohr effect. Cooperativity is observed with whole or half molecules and is mediated by divalent ions or high ionic strength. The O_2 equilibrium and kinetics of gastropod and arthropod haemocyanins were discussed. Undissociated *Panulirus interruptus* haemocyanin under conditions of cooperative O_2 binding was reported to show a non-autocatalytic oxygen dissociation behaviour (H. Kuiper, Groningen). Temperature-jump studies showed that the oxygenation reaction is heterogeneous in *Limulus* haemocyanin and its isolated subunits (C. Bonaventura, Duke University Marine Laboratory). The reverse Bohr effect in *Buccinum* haemocyanin was investigated by E. Wood (University of Leeds). The kinetic explanation for this effect was reported to be that the dissociation velocity constant was strongly pH-dependent whereas the association explanation was reported for a positive Bohr effect except that the pH dependency of the velocity constants goes in the reverse direction (M. Brunori, University of Rome).

The question of whether molluscan and arthropod haemocyanins are similar because of convergence or are homologous and have descended from a common ancestral gene remains unanswered. A. Ghirelli-Magaldi (Padova) showed that the available amino acid compositions of several arthropod and molluscan haemocyanins can be used to reconstruct a consistent evolutionary tree.

Since the first Haemocyanin Workshop in Naples 10 years ago, much progress has been achieved but nevertheless, the protein is proving to be more difficult to study than was first imagined. □

articles

Cyclic climatic variations in climate over the past 5,500 yr reflected in raised bogs

Bent Aaby

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Investigations of Danish raised bogs apparently indicate cyclic long term climatic variations with a periodicity of about 260 yr over the past 5,500 yr. The result could be used for modelling future climatic trends.

RAISED bogs represent a special type of peat bog: they are lens shaped, and highest in the centre. They receive no minerogenic water from their surroundings, but have their own water regime, with all of the moisture supplied from the atmosphere, as rain or snow. The humidity in the upper peat layers depends, therefore, essentially on precipitation, temperature and evaporation, a fact which makes raised bogs very sensitive to variations in climate.

Though past climatic changes are reflected in raised bogs as variations in the degree of decomposition (or humification) of the peat, interpretations are not straightforward. For example, a trend towards accumulation of more light coloured peat (decreasing humification) indicates increasing humidity in the bog, which may be attributable either to higher precipitation or lower temperature, or to a combination of the two. It is not possible to identify which of the two parameters is responsible, or to tell exactly how drastic or persistent a recorded climatic change may have been. The opposite shift, from light to dark coloured peat, does not necessarily depend only on climatic parameters, because peat formed under stable climatic conditions will also show the same trend in decompositional behaviour, since the increase in the relative distance to the water table changes as new peat accumulates. Shifts to drier bog conditions are therefore ignored as indicators of climatic changes.

Although changes from dark to light peat formation may indicate climatic shifts, local conditions, such as surface drainage patterns, may also affect patterns of peat formation. It is therefore important to investigate large open peat sections to distinguish widespread alterations (to be found at three or more places in a section) from local alterations. The surface of raised bogs from suboceanic areas comprises drier hummocks and wetter hollows. General variations in the water regime often cause an alteration in their distribution pattern (Fig. 1).

From the rather stationary centre the hummocks transgress in stable and/or dry periods and regress when conditions become generally wetter. A theoretical section, based on the Draved bog profile is shown in Fig. 1B. It can be demonstrated that Draved hummocks have existed at the same place for more than 2,500 yr, contrary to the theory of cyclical hollows/hummock succession (see, for example, ref. 1). Accordingly, an autonome succession between hollows and hummocks cannot have been responsible for the observed shifts in peat formation.

The hollow peat is more likely to reveal a detailed record

than the hummocks, where smaller variations in the humidity are not registered. Levels corresponding to the transgression and regression stages of the hummocks can be detected by measuring humification variations in the hollows.

Obtaining the material

I investigated a number of large, open peat sections in five raised bogs in Denmark. All of the bogs cover a rather extended area (more than 200 ha), and the sections are situated in the central part of the bogs. The degree of humification was measured by the colorimetric determination (see ref. 2). A humification curve is shown in Fig. 2, together with the relative frequency of two rhizopod genera. These genera occur in raised bogs connected to the moist part of the bog surface. Population frequencies reinforce indications of past changes in the water regime on the bog, as reflected in the humification curve.

Levels reflecting past climatic changes have been dated using the radiocarbon method. From Draved Mose a time/depth scale² based on 55 radiocarbon dates has been used. In the other cases one or two samples above and beneath levels in question have been dated. The ¹⁴C dates have further been calibrated to calendar years in accordance with the American bristlecone pine chronology (see ref. 3).

Figure 3 shows the dates of climatic shifts as determined in Danish bogs. Some dates are identical within statistical uncertainty, reflecting the general character of the climatic events. For example, the onset of colder and/or more humid weather conditions around AD 500 has been recognised in four different bogs⁴. The dates seem to be systematically distributed in time. Generally, there is a time interval of around 260 yr between palaeoclimatic shifts, as indicated in the left-hand side of the figure; but sometimes there is the double time span, 520 yr, between shifts. The reason could be that the intermediate climatic changes were too weak to be reflected in the bogs (for example, the missing shift around AD 1800, see Fig. 4), or that data are too sparse to show missing events. A few dates seem not to accord with the supposed periodicity (at ~ 1400 BC and ~ 2000 BC).

Although the raised bogs do reflect past climate, not every climatic shift is indicated in the peat structure because the rate of decay is rather slow. The degree of decomposition may therefore rather depend on general environmental conditions during an extended period⁵. When light, weakly decomposed peat was formed, wet periods were more frequent than dry ones, while opposite conditions prevailed when dark peat formation predominated. Raised bogs are therefore supposed to react as biological low pass filters only reflecting general long term tendencies

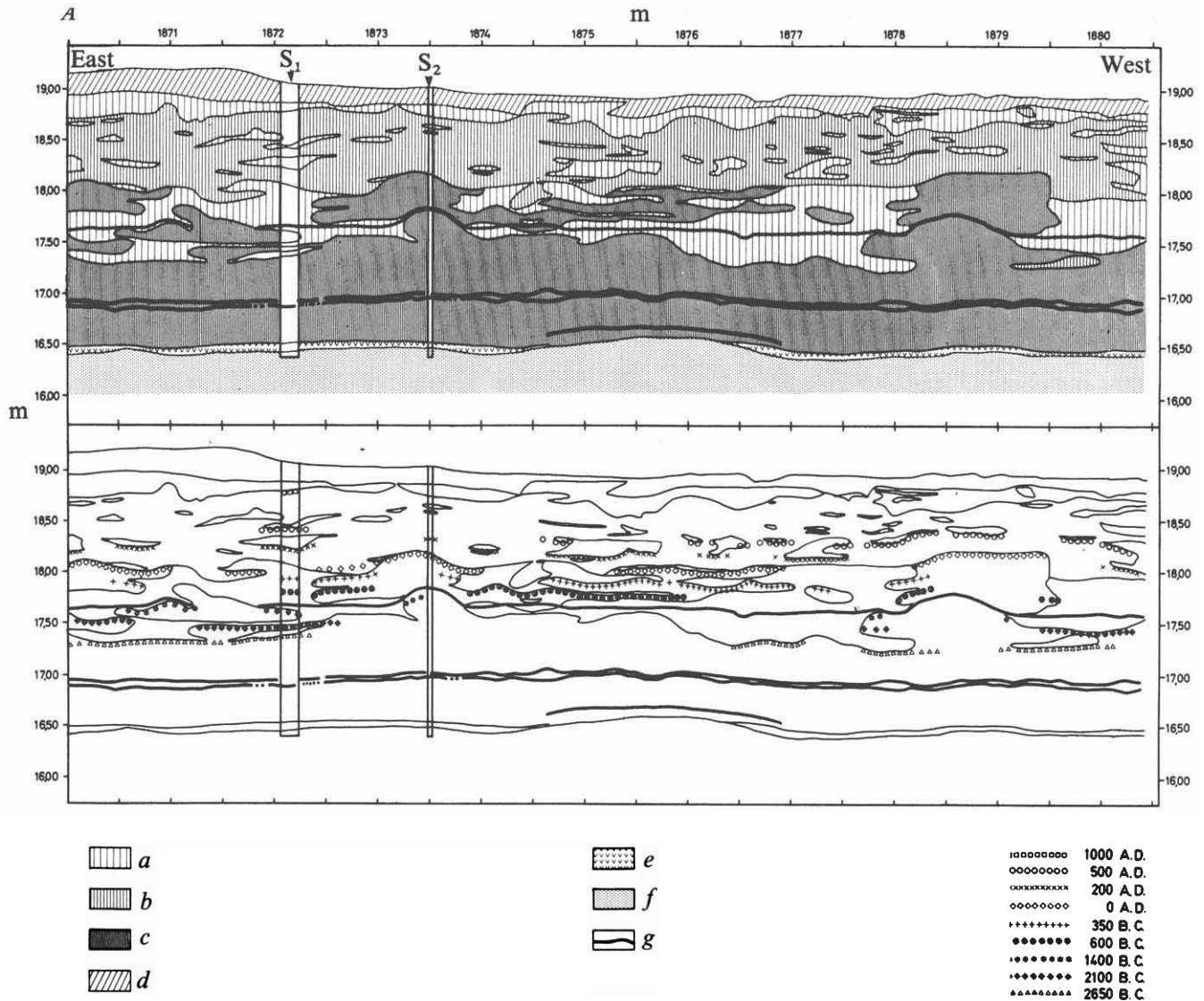
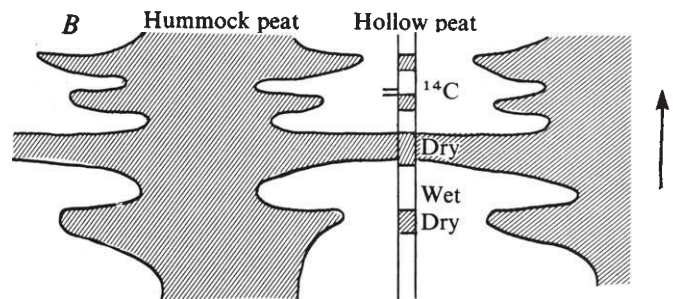


Fig. 1 A, Part of a 30-m long peat section from the central part of Draved Mose. The bog lies on aeolian sand. Peat formation began about 5500 bc. *a*, Slightly humified, sphagnum-vaginatum peat; *b*, moderately humified sphagnum-vaginatum peat; *c*, highly humified sphagnum-vaginatum peat; *d*, disturbed peat layer; *e*, hydromor; *f*, sand; *g*, charcoal layer; *s*, sampling sites. Top, visual variations in peat colour, together with distinct charred layers. Highly humified peat (*c*) dominates most of the section at about 1,873.5 m (at sampling site *S*₂) and at 1,879.0 m, where two hummocks have existed for more than 2,500 yr. From the rather stable centre the hummocks transgress in dry periods and regress when it becomes generally wetter (see *B*). Bottom, Approximately synchronous levels. Based on the three distinct charred layers (two lying close together at 16.90–16.95 m and one at 17.60 m) and 55 radiocarbon dates from sampling site *S*₁, the bog development in this area has been reconstructed. General changes from dark to light peat formation occur nine times (see the symbols). The dates of changes are given rounded off to



the nearest 50 yr. **B**, Theoretical peat section, showing that the hummock area increases in dry periods, and becomes smaller in wetter periods.

in climate, and the 260-yr cycle seems to be the shortest periodicity revealed.

This interpretation of changes in the degree of humification is supported by historical evidence from the past millennium. From England a temperature curve for the past 1,100 yr is available (Fig. 4). If the curve is smoothed by a 200-yr low pass filter it shows the same general climatic trend reflected in the Danish bogs. The latest period of colder/wetter climate previously detected from raised bogs occurred during the first part of the sixteenth century, that is, at the beginning of a temperature decline corresponding to the onset of the "little ice age". The preceding change in peat formation patterns (in the middle of the thirteenth

century) seems to correspond to the end of the mediaeval warm periods. The English temperature curve before AD 1100 is rather uncertain (H. H. Lamb, personal communication) and cannot be used for control purposes. Ice-isotope investigations, however, indicate that colder conditions prevailed around AD 1000, as do the Danish bogs. Younger variations from dark to light peat formation thus apparently reflect climatic changes and the same is probably true for older peat variations. Figure 4 also shows that there may be little (probably less than 50 yr) or no time lag between dated humification changes and the beginning of long term climatic trends.

Following the most recent humification change during

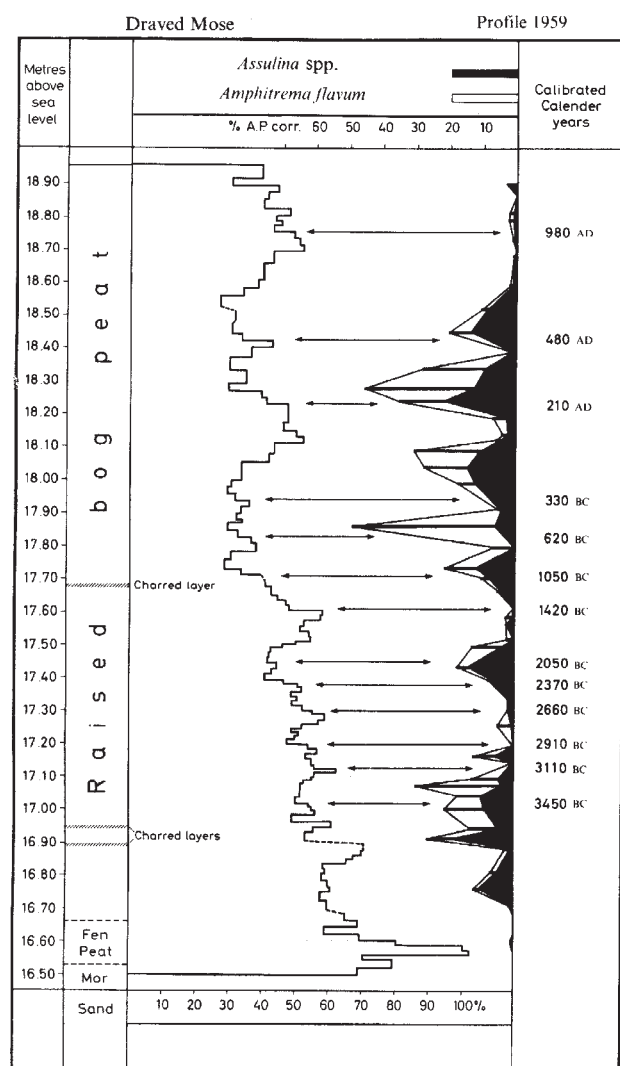


Fig. 2 Degree of humification and content of two rhizopod genera in a vertical peat column in hollow peat from Draved Mose (S₁, Fig. 1). Arrows, Levels assumed to reflect climatic shifts.

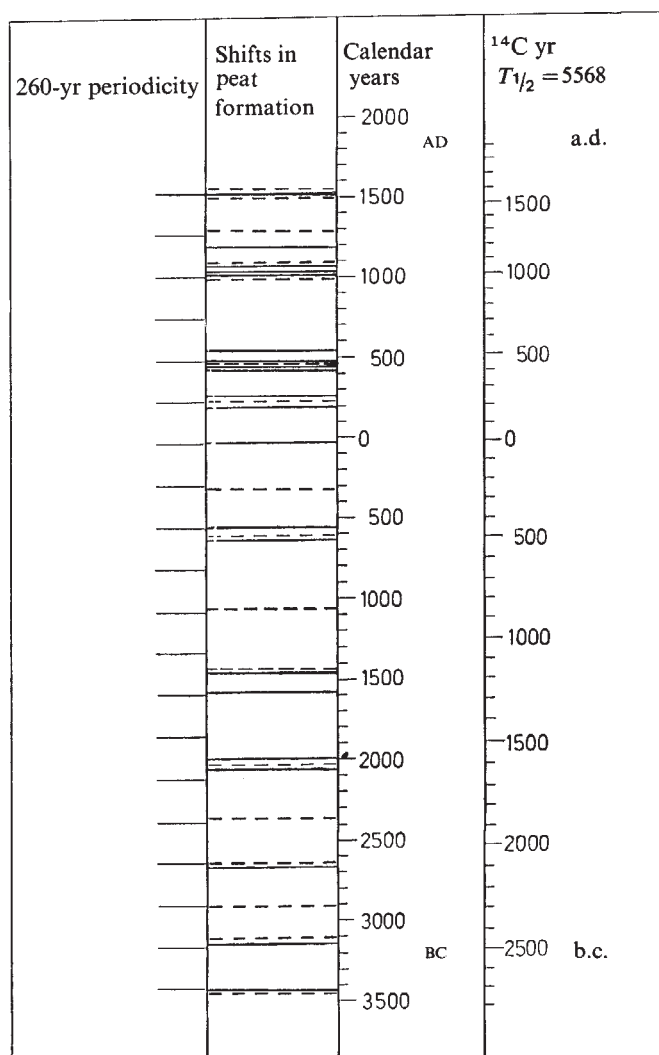


Fig. 3 Record of climatically conditioned shifts from dark to light peat formation, derived from sections in 5 Danish raised bogs. Dotted lines, dates of changes reflected in Draved Mose; full lines, results from other bogs.

the first part of the sixteenth century, another change should have occurred during part of the eighteenth century. It may indeed be noticed that a decrease in temperature occurred at that time on the England temperature curve (Fig. 4). That cooling trend was distinctly weaker than preceding ones in the thirteenth and sixteenth centuries, and was perhaps too small to leave traces in the Danish bogs.

Statistical calculations

A statistical test was made to check the significance of the apparent periodicity of humification patterns, and also to determine more accurately the length of the period.

Groups of dates in Fig. 3 differing by less than the statistical uncertainty of the datings have been considered as reflecting the same climatic events, dated to an arithmetic mean. This procedure reduces the number of events under consideration from 39 to 17, each having a smaller statistical dating uncertainty than if the dates were considered independently.

Series of dates were established with constant time intervals $T=250$, 260 and 270 yr, respectively. For each value of T the phase of the series was chosen so as to minimise the deviation of the observed dates of events from the expected dates; for example, AD 1520 was used as the youngest date in the series $T=260$ yr. It was assessed

Table 1 Distribution tests of dates indicating climatic changes (see Figs 3 and 5b) from three cyclic periods*

	250 yr	260 yr	270 yr
Uniform distribution test	Accepted	Refused	Moderately accepted
Significance level	20%	$\leq 1\%$	9%
Normal distribution test		Accepted	Moderately accepted
Significance level		77%	5%
Minimised standard deviation		± 41 yr	± 74 yr

* Dates lying close together in time are supposed to reflect the same variation in climate and are only taken as a single event, dated to the arithmetic mean of the dates, which minimises standard deviations.

whether the deviations from the three periods were uniformly distributed over the entire interval. Table 1 shows that this seems to be the case for $T=250$ years, indicating no periodicity of that length.

The deviations in the two other series may show a systematic variation, and the probability that they are normally distributed has been tested (Table 1). Both the normal distribution test and the standard deviation test identify $T=260$ yr as a significant periodicity in the original series of events (Fig. 5). The standard deviations on the calibrated radiocarbon dates were not included in the calculations, but it seems likely that the calculated standard deviation in Table 1 is of the same order as, or slightly smaller than, the standard deviation of the individual calendar dates. This may indicate that in most cases the standard deviation is attributable to the uncertainty in the dating method. I thus propose that a major long term fluctuation in climate has had a rather distinct cyclicity with a periodicity of about 260 yr during the past 5,500 yr in north-western Europe.

Fig. 4 Relationship between long term trend of temperatures in central England, shown as 50-yr means, and dates of climatic shifts indicated by bog investigations (full lines). Temperature curve based on instrumental observations back to AD 1700, and before that time on historical records; the oldest part is rather uncertain (H. H. Lamb, personal communication). The curve has been smoothed by a 200-yr low pass digital filter (see refs 5 and 6). An expected change in peat formation around AD 1780 was not found in the bog profiles and is shown as a dotted line.

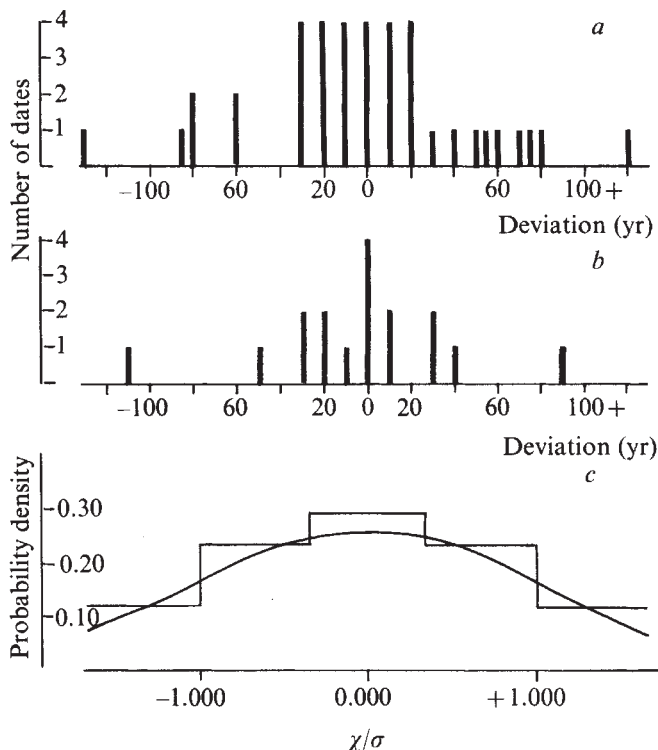
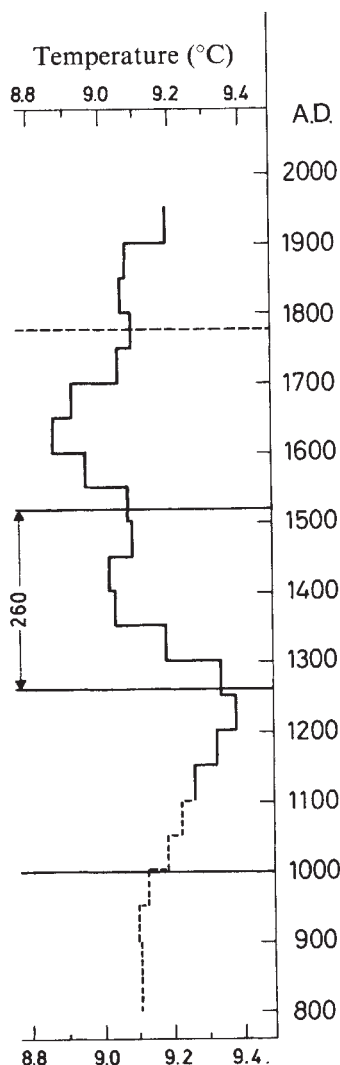


Fig. 5 *a*, Deviation (yr) from a climatic periodicity of 260 yr, beginning in AD 1520 (see Fig. 3). *b*, The same calculations based on the assumption that shifts dated to almost the same time (using ^{14}C analysis) are strictly synchronous and dated to the mean of the dates. *c*, The actual distribution of the 17 deviation dates from *b* compared with the normal distribution curve having the same mean and variance.

Future climate

To demonstrate periodicity, a time span of generally more than 10 times the length of the individual periods concerned must be considered. In this investigation the time interval has been extended to about 5,500 yr, or more than 20 times the length of the period in question. It is therefore justifiable to use the observed periodicity to predict future climatic trends. This suggests that a general trend towards decreasing mean temperatures and/or wetter conditions may begin in the last part of this century or in the first part of the twenty-first century in north-western Europe.

Predictions of long term trends in climate have previously been based on ice-isotope⁷ and tree-ring^{8,9} data from the past millennium, and even though these predictions essentially agree, caution must be applied in interpretation particularly as a physical explanation for climatic changes is still lacking. Pollution of the atmosphere may turn out to be a dominating factor in the future¹⁰. Perhaps resulting in climatic trends different from projected natural trends.

On the other hand, the Danish bog data could contribute to the design of an atmospheric model sufficiently reliable to provide some hints as to the future trends.

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Complementary base pairing and the origin of substitution mutations

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On the basis of chemical considerations and model building, the Watson-Crick concept of complementary base pairing is extended to a wider range of DNA pairs than A-T and G-C (including A-C, G-T, A-A, G-G and G-A) by invoking imino or enol tautomers (or protonated species) and syn isomers. The virtual absence of these additional base pairs from DNA is explained in terms of the low frequency with which these unfavoured forms occur and the two-step mechanism of DNA synthesis, whereby residues are first incorporated by the DNA polymerase and then checked. This base-pairing hypothesis is used to explain the origin, nature and level of spontaneous substitution mutations, their enhancement by base analogues, and the unique effects of certain mutator alleles.

ALTHOUGH the bi-helical complementary base-paired structure of DNA implies mispairing events as the principal means for generating substitution mutations, a general mechanism for mispairing has not emerged. Watson and Crick did suggest as part of their formulation of replication that spontaneous mutations might be due to the occasional occurrence of a pu-pyr pair with a base in one of its less likely tautomeric forms¹. But while plausible, this suggestion fails to explain the observed frequencies of spontaneous substitution mutations, 10^{-9} to 10^{-12} per base pair synthesised², simply from the estimated frequencies of unfavoured tautomers in solution, 10^{-4} to 10^{-5} (refs 3-6). Moreover, while pu-pyr mispairing events involving minor base tautomers have been conceived as a qualitative rationale for the origin of transitions, a comparable set of mispairs leading to transversions has not been proposed. A mispairing rationale for the mechanism of many chemical mutagens is also wanting.

We describe here a general base-pairing hypothesis that explains both qualitative and quantitative features of substitution mutations. This hypothesis is based on well recognised chemical features and isomeric equilibria of nucleotides and the notion that the expression of these equilibria accounts for the level at which mispairing events occur. The hypothesis depends on two principal assumptions. The first is that there is a wider set of complementary base pairs than the two proposed by Watson and Crick⁷, A-T and G-C, that are compatible with the steric constraints of a regular DNA helix (Fig. 1). The non-Watson-Crick complementary pairs constitute mispairs of two types, pu-pyr, which can give rise to transitions, and pu-pu, which can lead to transversions. The frequency with which such mispairs occur is due mainly to the manifestation of the equilibrium constants for the isomerisation processes required for their formation, that is, keto-enol and amino-imino tautomerisation of the base moieties for the pu-pyr mispairs and both such tautomerisation and anti-syn isomerisation about the glycosyl bond of the nucleoside moiety^{8,9} for the pu-pu mispairs. These two classes of mispairs can give rise by single mispairing events to all conceivable substitution mutations involving the commonly occurring nucleotides in DNA (Table 1).

The second major assumption is that there are two opportunities to express the relevant isomeric equilibria involved in

particular mispairing events during the process of adding a base pair, first during catalytic incorporation of the new base on the growing strand, and again during a checking step. This results in the relevant equilibria limiting the frequency of mispairing to the level seen in biological systems.

Using these two assumptions, we are able to explain a wide range of mutagenic phenomena, including: how mispairing for each conceivable type of substitution mutation can lead to the frequencies observed *in vivo*; the rate observed for transitions and transversions¹⁰; the origin of the unidirectional effect of the Treffer's mutator gene on the A-T→C-G transversion^{11,12}; and the specificity of the base analogues 5-bromouracil (5-BrU) and 2-aminopurine (2-AP) in inducing transitions but not transversions^{13,14}. In what follows, we present the essential features of the hypothesis and the way in which it explains these basic phenomena of mutagenesis. A preliminary report of this work has been made¹⁵.

Chemical plausibility of proposed base pairs

Each of the proposed mispairs with the H-bonding schemes indicated in Fig. 1 was studied by model building and shown to be sterically compatible with a Watson-Crick helix. Moreover, base oppositions corresponding to each of these mispairs have been shown experimentally to take up intrahelical conformations in long polynucleotide helices dominated by Watson-Crick pairs (ref. 16 and M.D.T., H. Chen and J. R. F., unpublished). By contrast, those base oppositions not invoked for mispairing events (pyr-pyr), were experimentally found to take up only extrahelical conformations in such polynucleotide helices (refs 16, 17 and J. Balcerski, M.D.T., and J.R.F., unpublished). Moreover, these oppositions could not be satisfactorily built into a regular DNA helix as base pairs. The proposed pu-pyr mispairs conform precisely to Watson-Crick helix geometry, whereas the proposed pu-pu mispairs require a slight ($\sim 9^\circ$) distortion of the glycosyl bond angle of the base in the syn configuration without any effect on the glycosyl bond separation distance. Distances between H-bonded atoms are standard. Finally, there is much chemical and crystallographic evidence for the occurrence in certain small molecules of the invoked unusual base tautomers^{3-6,18} or nucleoside isomers (reviewed in ref. 19).

Mispairing and DNA synthesis

DNA synthesis seems generally to be a two-step process, involving an incorporation reaction, followed by a checking reaction²⁰. In the cases of bacteriophage T4 (ref. 21) and *Escherichia coli*²² at least, both these catalytic activities reside in a single polypeptide. We assume that the synthetic element of these polymerases does not directly sense the incoming base; instead, the whole DNA synthetic apparatus, consisting of several different proteins in addition to the polymerase^{23,24} confers a fixed orientation on the immediate template residue and fixes the orientation and the glycosyl bond separation distance at the growing strand end. Covalent incorporation of a complementary nucleotide is seen as involving nucleoside triphosphate binding to both the polymerase on the synthetic apparatus and the geometrically constrained template residue in a specific way that is determined by H-bonded base pairing.

This binding of monomer is followed by catalysis of phosphodiester bond formation as a natural consequence of complementary base pairing, since only then is the polymerase catalytic site and the 3'-OH of the growing strand in position to attack the α - β pyrophosphate bond of the nucleoside triphosphate. The polymerase-catalysed reaction therefore incorporates any nucleotide when its base is in an isomeric form that allows a complementary pair and discriminates effectively against all others. This mechanism presupposes that once productive pairing or mispairing occurs, isomeric re-equilibration is prevented because the synthetic apparatus, perhaps by inhibiting the breathing of base pairs or by excluding water, makes the H-bonding donor and acceptor sites of the bases inaccessible to the water required for tautomerism. Thus, not only the Watson-Crick base pairs, A-T and G-C, will be incorporated, but also the complementary mispairs, which after another round of replication show themselves as substitution mutations (Table 1).

At this stage, mispairs involving only unfavoured tautomers will have occurred at a level reflecting the sum of the frequencies of unfavoured triphosphate tautomers and of such tautomers in the template residue (since equivalent A-C and G-T pairs occur when either the template or the monomer residues are in the unfavoured tautomeric form). By contrast, mispairing involving syn isomers will occur at a level reflecting the product of the frequencies of the required unfavoured tautomer in the template strand and the syn nucleoside triphosphate. Syn isomers are forbidden in the template strand since, at the outset, those residues are anti in a regular DNA helix⁷, and both the stacking in the single-stranded template segment and the rigidity imposed by the synthetic apparatus must create a large barrier to rotation about the glycosyl bond. Thus, those mis-

pairings that lead to transitions will have been introduced to a level of 10^{-4} to 10^{-5} (the frequency of unfavoured tautomers), while those leading to transversions will have been introduced to a level of 10^{-4} to 10^{-5} times $\sim 10^{-1}$ if the syn base is G^{8,9} or times $\sim 5 \times 10^{-3}$ if it is A^{8,9}. Thus, the fidelity in this synthetic step is greater for transversions than for transitions. These values are, of course, far greater than the biological levels of mutation.

Mispairing and the checking step

Once incorporated, a base pair must be checked to increase the level of fidelity by some mechanism that can remove mispairs with great efficiency. A mechanism by which this might be achieved is suggested by the occurrence of a 3'-5' exonuclease activity in the polymerase moieties of bacteriophage T4 and *E. coli*^{21,22}, which apparently excises unpaired residues at the growing strand terminus²³. In higher species there are indications that this activity may be relegated to a separate molecular entity²⁶.

It is reasonable to assume that the isomeric equilibrium constants are about the same for the residues of the new terminal base pair as for the triphosphates and for the template residues. In the interval between release of the new base pair by the polymerase and the coming into position of the 3'-5' exonuclease moiety, there is renewed opportunity for breathing and accessibility of solvent. Therefore, we expect for transitions the same isomeric equilibria manifest in the synthetic step to be expressed once again during the checking reaction. The checking of transition mispairs should thus lead to a squaring of the fidelity level of the synthetic step, that is, a total fidelity of 10^{-8} to 10^{-10} . For transversions, however, the situation is more complex. For this class of substitutions, also, tautomeric re-

Fig. 1 The complementary base pairs that can be formed from the DNA bases A, G, T, and C are illustrated. The isomeric forms of the bases that predominate in 'physiological' conditions allow only the two Watson-Crick pairs (*a, b*). Tautomerism involving only one proton transfer gives rise, however, to two equivalent pairing schemes each for the pu-pyr pairs, A-C (*c, e*) and G-T (*d, f*) and one scheme each for the pu-pu pairs A-A_{syn} (*g*), G-G_{syn} (*h*), A-G_{syn} (*i*) and G-A_{syn} (*j*). There is no way of constructing a complementary pyr-pyr pair. Note also that the glycosyl bonds of pairs *a-f* are twofold symmetry related regardless of which strand is the fixed template, whereas pairs *g-j* are complementary only when the anti residue is on the template. The enol form of either G or T leads to a G-T pair with three H bonds (*d, f*), whereas the imino form of either A or C makes for A-C pairs with two H bonds (*c, e*). The imino form of A also makes possible A-G (*i*) and A-A (*g*) pairs with two H bonds, provided that the second base G and A, respectively, is in the syn configuration. It should be noted that the A-G pair shown in (*i*) can also be achieved by protonation of the A residue at N1 without tautomerism to the imino form at approximately the same cost in energy. Base pairs involving two H bonds are possible between a tautomerised G and either A_{syn} or G_{syn} (*j, h*). Here, the conventional enol tautomer of G may give rise to a close contact between a 2-amino hydrogen of G and the C8 hydrogen of the opposing A_{syn} or G_{syn}, or else this C8 hydrogen may be restricted from H bonding to water. Therefore, a chemically possible minor tautomeric form of G is used instead, an enol, imino tautomer formed by transfer of a 2-amino hydrogen to the 6-oxygen to achieve G-G_{syn} and G-A_{syn}.

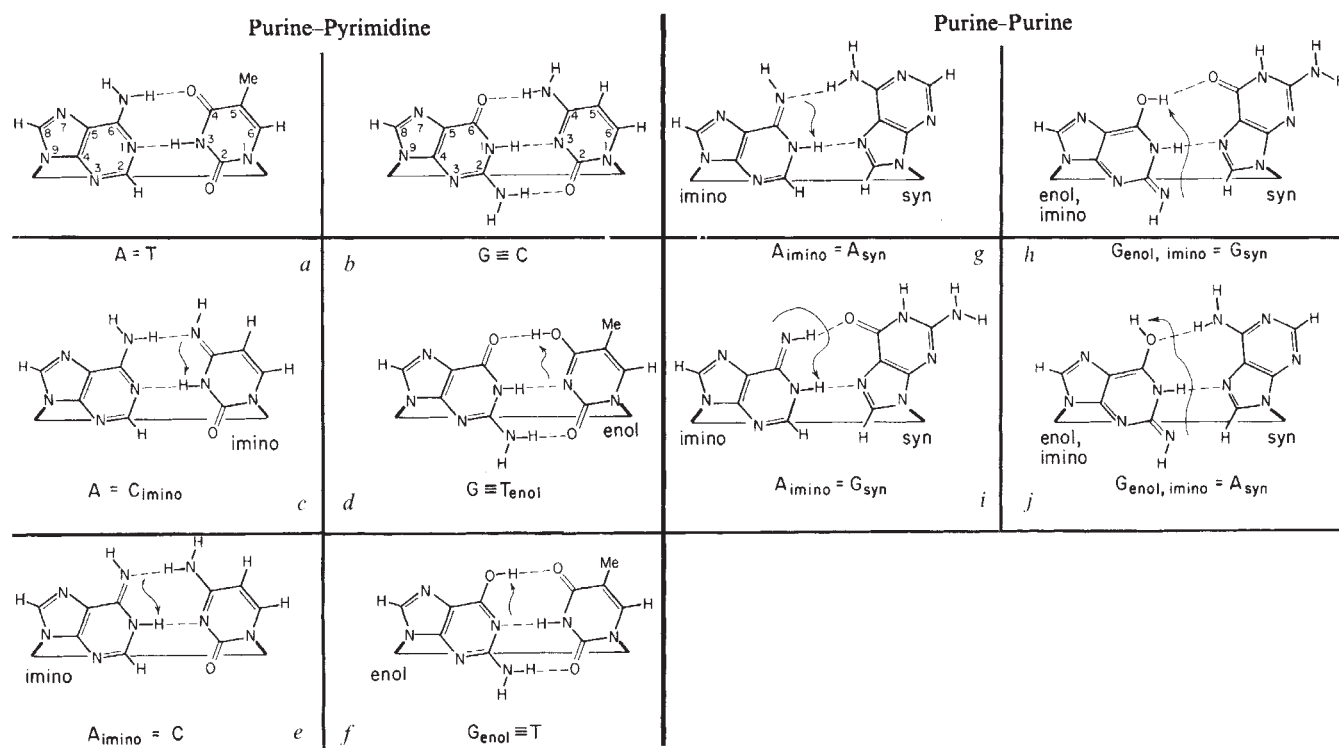
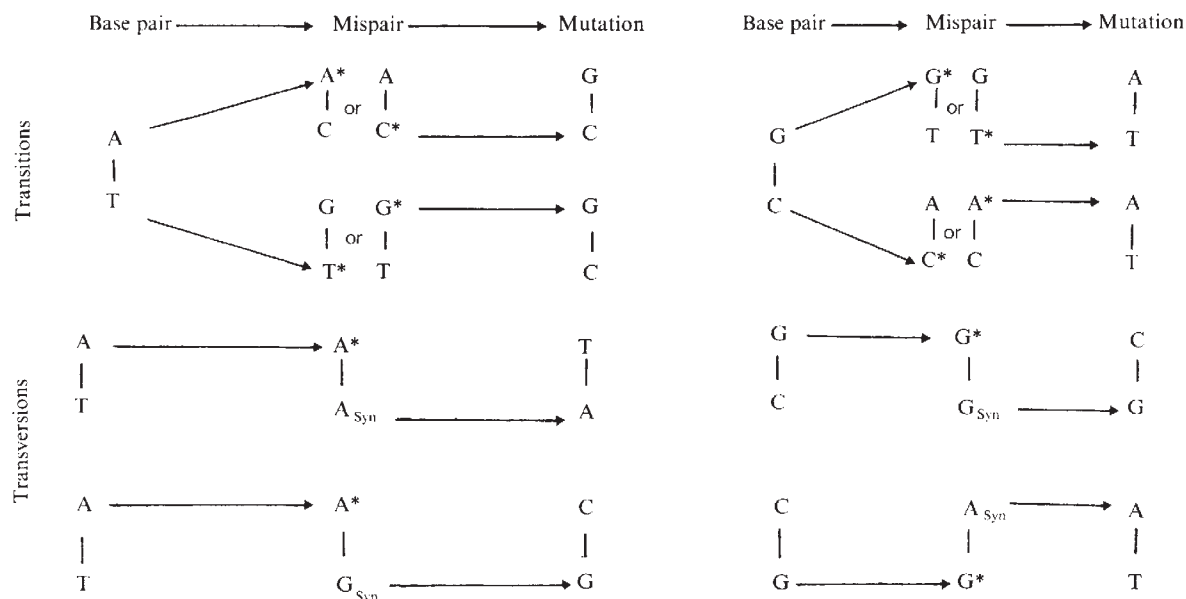


Table 1 Pathways for substitution mutations



Pathways are shown whereby every conceivable transition and transversion can occur by single mispairing events that each involve a non-Watson-Crick complementary base pair. Note that whereas transitions can occur with each strand of a base pair, transversions are viewed as occurring only on the strand with the purine template residue since pyr-pyr mispairs are not believed to occur with any significant frequency.

*, Base in a minor tautomeric form; syn. the unfavoured configuration about the glycosyl bond of that residue (see Fig. 1). Arrows indicate rounds of replication.

equilibration of template residues is expected in the checking step; but re-equilibration of the syn configuration of the terminal residue is unlikely because the activation energy for rotation about the glycosyl bond of an incorporated residue stacked on its neighbour should be much larger than it is in the entering triphosphate. Consequently, the exonuclease step should enhance fidelity only by the frequency of the minor tautomer in the template residue, that is, by another factor of 10^{-4} to 10^{-5} . Therefore, the total fidelity for transversions will be less than the square of that achieved in the first step, amounting to 10^{-9} to 5×10^{-12} , which is nevertheless greater than that for transitions. It follows that in systems lacking the exonuclease function, substitution mutation rates will be as expected from the first step alone.

Evaluation of the hypothesis

In evaluating the hypothesis, we have considered the literature on nucleic acid chemistry, on the specificity of *in vitro* and *in vivo* nucleic acid biosynthesis and on the mechanism of mutagenesis. We have not found any experimental observations that could not be interpreted in terms of our two principal assumptions. Here, we describe three important types of observations on substitution mutations that illustrate the consistency between hypothesis and observation.

(1) One of the major probes of the mechanisms of mutagenesis involves studies with compounds that induce or enhance the level of spontaneous mutations. From the work of Benzer and Freese, in particular, it has been established that certain base analogues are highly specific in inducing transitions only^{13,27}. For example, pyrimidines such as 5-BrU, which exhibit a greater tendency to enolise than does their natural counterpart, thymine, behave this way. This selective effect is exactly as expected from our hypothesis since transitions are mediated only by pu-pyr mispairs, whereas transversion mispairs do not involve pyrimidine residues.

2-AP is another base analogue that induces only transitions^{13,14}. The inability of this purine to induce transversions is of particular interest in view of our proposal that transversions are brought about only by pu-pu mispairs.

The way in which this analogue induces transitions is not difficult to rationalise. Although lacking a substituent on C6, it can interact "normally" with T on the template to form a complementary pair with two H bonds. By tautomerism to the imino form, it can "mispair" with C also by way of two H bonds. The alternative mispairing scheme with C, involving the amino tautomer of 2-AP seems less likely because it allows only one H bond, and the H-bonding tendencies of the internal N1 of the purine and N3 of the pyrimidine would be left unsatisfied.

The lack of potential for pu-pu mispairs involving 2-AP can

Fig. 2 Potential of 2-AP for pu-pu pairing. The inability of 2-AP to form transversion mispairs is illustrated. In the syn configuration, 2-AP can form no H bonds with A (a), and whereas a pair with one H bond might be formed with G, it results in a bad Van der Waals' contact (b). When in the anti configuration, 2-AP can make but one H bond with either A (c) or G (d), and then only by tautomerising to the less favoured imino form.

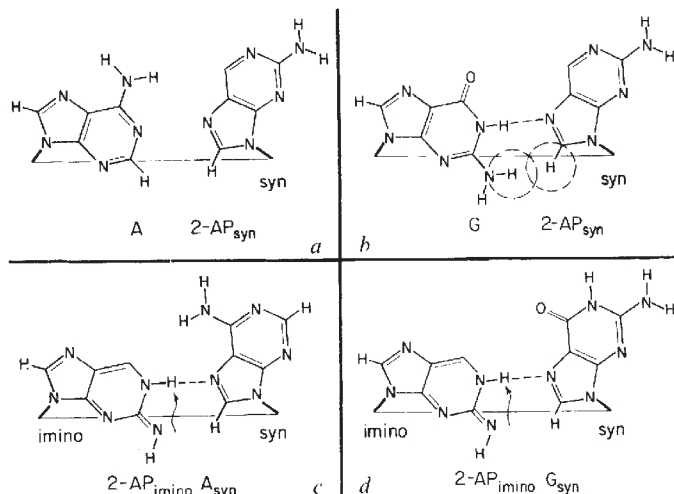


Table 2 Comparison of observed frequencies and calculated mutation rates

	Base pair substitution	Mutator allele*	Observed† reversion frequencies		=	Predicted reversion rates‡				No. of pathways
				Total		Synthetic step	×	Checking step	×	
Transitions	AT→GC	+	5×10^{-9} – 4×10^{-10}	5×10^{-9}	=	(5×10^{-5})	×	(5×10^{-5})	×	2
		D5	$(1-2) \times 10^{-6}$	1×10^{-4}	=	(5×10^{-5})	×	(1)	×	2
	GC→AT	+	$(3-9) \times 10^{-10}$	5×10^{-9}	=	(5×10^{-5})	×	(5×10^{-5})	×	2
		D5	$(2-4) \times 10^{-5}$	1×10^{-4}	=	(5×10^{-5})	×	(1)	×	2
Transversions	GC→CG	+	8.6×10^{-10}	2.5×10^{-10}	=	$(5 \times 10^{-5})(1 \times 10^{-1})$	×	(5×10^{-5})	×	1
		D5	2.9×10^{-6}	5×10^{-6}	=	$(5 \times 10^{-5})(1 \times 10^{-1})$	×	(1)	×	1
	AT→TA	+	3.9×10^{-9}	1.2×10^{-10}	=	$(5 \times 10^{-5})(5 \times 10^{-2})$	×	(5×10^{-5})	×	1
		D5	$(2-3) \times 10^{-6}$	2.5×10^{-6}	=	$(5 \times 10^{-5})(5 \times 10^{-2})$	×	(1)	×	1
	CG→AT	+	$(3-5) \times 10^{-9}$	1.2×10^{-10}	=	$(5 \times 10^{-5})(5 \times 10^{-2})$	×	(5×10^{-5})	×	1
		D5	3×10^{-6} – 2×10^{-6}	2.5×10^{-6}	=	$(5 \times 10^{-5})(5 \times 10^{-2})$	×	(1)	×	1
	AT→CG§	+	2×10^{-8} – 2×10^{-9}	2.5×10^{-10}	=	$(5 \times 10^{-5})(1 \times 10^{-1})$	×	(5×10^{-5})	×	1
		D5	5×10^{-6} – 3.1×10^{-7}	5×10^{-6}	=	$(5 \times 10^{-5})(1 \times 10^{-1})$	×	(1)	×	1
	AT→CG§	+	$(6-9) \times 10^{-10}$	2.5×10^{-10}	=	$(5 \times 10^{-5})(1 \times 10^{-1})$	×	(5×10^{-5})	×	1
		D5	1×10^{-7} – 5×10^{-8}	5×10^{-6}	=	$(5 \times 10^{-5})(1 \times 10^{-1})$	×	(1)	×	1

Comparison of frequency of different substitution reversions in the tryptophan synthetase *A* gene of *E. coli* (data taken from Fowler, *et al.*¹⁰) with theoretical mutation rates calculated from characteristics of proposed non-Watson-Crick complementary base-pair intermediates.

*+ wild type; D5, conditional mutator gene, to which we assign the phenotype, exonuclease⁻.

†Ranges are given to encompass several sets of data obtained for the same mutant by Fowler *et al.*¹⁰.

‡For the purposes of these calculations, a value of 1×10^{-1} is taken for the equilibrium frequency of G_{syn} and 5×10^{-2} for that of A_{syn} . The same equilibrium frequency, 5×10^{-2} is assumed for all unfavoured tautomers. If this were actually the case, these calculations would have to take into account that the A-C and G-T pairs each could occur with either the template or the monomer residues in the unfavoured tautomeric form; then, the calculated rates of transitions would have to be raised by a statistical factor of four (two for each step). It is much more likely, however, that the tautomeric equilibria differ for the two members of each pair and differences as small as two-threefold effectively reduce this factor to one. With only one unfavoured tautomer in pu-pu pairs, this factor is also one for transversions.

§These represent the same type of transversion at two different sites within the same gene.

be seen (Fig. 2) to arise from its lack of any substituent on C6. For incorporation opposite a purine, 2-AP must enter in the syn configuration, in which case it can form no H bonds with A (Fig. 2a). Against G, the possibility of only one H bond as well as the potential close contact between the C2 amino of the template residue and the C8 hydrogen of 2-AP, which can also interfere with solvation of the C2 amino group, prohibits that pairing as well (Fig. 2b). Further, once incorporated, the amino tautomer of 2-AP in the anti configuration of the template strand cannot form any H bonds with either A_{syn} or G_{syn} triphosphates, while the imino tautomer can form pairs which will allow but one H bond (Fig. 2c and d), which would seem insufficient. Purine analogues that favour syn configurations and have appropriate substituents are, however, expected to induce transversions; and purine analogues of that type which also have a more favourable capacity to tautomerise than A or G should enhance the rate of both transitions and transversions. It may similarly be possible to explain the mutagenic effects of reactive substances that modify bases of intact DNA, thereby altering their capacity either to tautomerise (for example, hydroxylamine^{28,29} and Cu^{2+} (ref. 30)) or to be fixed in a syn configuration. Since many such reactive compounds are also carcinogens³¹, a correlation is suggested.

(2) Among mutator genes, there is a striking example discovered by Treffers¹¹ of an allele in *E. coli* that selectively enhances the rate of the A-T→C-G transversion but not the reverse transversion¹². A consideration of the pu-pu mispairs for these two transversions (Fig. 1,j) and the restriction that the syn residue of those pairs be the entering one, suggests how the unidirectional effect of this mutator comes about. Whereas the forward transversion must be mediated by A_{imino} - G_{syn} , the reversion can only be mediated by $G_{enol,imino}$ - A_{syn} and not by the mispair by which it is formed (Table 1). The physiological mechanism by which this mutator is expressed can be imagined to involve a gene coding for some component of the replicative apparatus that allows incorporation of G_{syn} but not A_{syn} . (This would also result in parallel discrimination between the A- A_{syn} and the G- G_{syn} mispairs.) Obviously, a mutator with this kind of discrimination could not affect the rate of transversions because only anti isomers participate in transition

mispairs. The discriminatory characteristics of the Treffers' mutator and others^{32,33} clearly correlate with the proposed features that distinguish the mispairs that give rise to transitions from those that lead to transversions.

(3) The rates of transitions and transversions are also consistent with our proposed base-pairing schemes. Specific data against which to evaluate the mechanisms proposed for substitution mutations are provided by a study¹⁰ of spontaneous substitution mutation frequencies for particular sites on the tryptophan synthetase *A* gene of *E. coli*¹⁴, for which the base-pair changes are known. Those frequencies, determined both in the absence and presence of a mutator gene, *mutD5*, are shown in Table 2. Presented for comparison are the rates we calculate for each transition and transversion from the relevant isomeric equilibria for the proposed complementary non-Watson-Crick mispairs. (For this comparison, it is assumed that *in vivo* mutation rates and the observed frequencies they generate are equivalent. In fact, the observed values should be high, but by no more than a factor of five³².) Because of the availability of two mispairing routes to both transitions, but only one to each transversion (see Table 1) the rates calculated for the transitions (only) contain a factor of two. In the case of *mutD5* we make the explicit assumption that this mutation, though not in the exonuclease gene³⁴, has inactivated the checking function. The obvious correlation that this assumption allows us to make between the observed frequencies and the rates calculated, reinforces our confidence in the assignment of the *mutD5* phenotype. Thus, it can be seen that the agreement between the observed reversion frequencies for the two transitions examined in the presence of *mutD5* and our estimate for the synthetic step alone is satisfactory; so is that between the frequencies measured in the absence of *mutD5* and our calculated rates. Furthermore, the experimental data agree with our expectation that the total fidelity must approximately equal the square of the frequencies of the unfavourable tautomers only for transitions, where the complementary intermediates are A-C and G-T pairs.

The complexity postulated for transversions is also evident. As demanded by our mechanism and consistent with separating the *mutD5* frequencies from the reversion frequencies observed

in the absence of this mutator, greater fidelity can be seen to be achieved for transversions in the synthetic step than in the checking step. This is because syn isomers should have no role in the checking step. The *mutD5* frequencies are also consistent with our expectation that the synthetic step achieves greater fidelity for transversions than for transitions because of the involvement of syn isomers as well as unfavourable tautomers in transversion mispairs. Indeed, agreement between the observed frequencies in the presence of *mutD5* and the rates calculated for the synthetic step alone for all transversions is quite close, considering that the syn-anti equilibria must be taken into account. Nevertheless, it is apparent that the overall fidelity observed for transversions is somewhat less than we expect. Conceivably, this is because of a lower efficiency in removal of syn than anti residues by the exonuclease. Such a differential effect towards syn compared with anti residues has been observed for other exonucleases³⁵.

The agreement in both trends and levels between observed and calculated values for the various substitution mutations is encouraging. Nevertheless, we recognise that frequencies are being compared with rates and that the calculations do not take into account such modulating effects as nearest neighbour interactions (which could account for the frequency differences observed in the two cases of the same transversion A-T→C-G at two different loci shown in Table 2) and differences in the relative stability of the various mispairs. Since the calculated rates follow the observed trend, these modulating effects must be small relative to the major factors that we have introduced in the calculations.

The hypothesis we have presented here concerning a new set of complementary base pairs and their relevance to mutagenesis is chemically reasonable and consistent with a large body of biochemical and genetic facts. We therefore believe that the main features of these concepts are correct, though the details may require modification. Experiments explicitly designed to test particular features of the hypothesis are needed to assure their validity. We are initiating some, and we hope that others will be prompted to design additional tests.

The wider concept of complementary base pairing that has been introduced here has relevance to other problems in molecular biology. In the accompanying paper³⁶, we address the

question of their role in protein synthesis. Later reports will deal with their relevance in other contexts.

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Base pairing and fidelity in codon-anticodon interaction

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Base pairing in codon-anticodon interaction has been investigated in order to understand the basis on which particular base pairs have been selected for or against participation at the wobble position and the basis for codon-anticodon infidelity.

STUDIES of the genetic code revealed that the Watson-Crick pairing rules for DNA¹ are only followed strictly for the first two base pairs of codon-anticodon interaction². Much of the pairing at the third codon position also involves the normal Watson-Crick base pairs, A-U, and G-C, but for several codon-tRNA interactions, non-Watson-Crick pairs are clearly required. This led Crick to propose that the pair at the 3' end of the codon can deviate (from complementary helix geometry) or "wobble" within arbitrary limits (to the glycosyl bond separation distance and dihedral angles) that are consistent with the additional pairing interactions suggested by the code.

Our reconsideration of this problem takes into account the

chemical features of the bases and the stereochemistry of non-Watson-Crick complementary base pairs, described in the accompanying paper³, that have allowed us to suggest mechanisms for mispairing in replication. We deduce that the "wobble" pairing schemes for G-U, U-G and U-I must be as proposed by Crick⁴, but that an alternative base-pairing scheme for A-I is a more suitable codon-anticodon interaction. We also show that in a manner similar to the way they act in nucleic acid biosynthesis³, complementary non-Watson-Crick base pairs contribute to the natural level of infidelity in protein synthesis. A preliminary report of this work has been made⁵.

Base-pairing alternatives

The wider set of complementary base pairs described in the accompanying paper³, utilises base tautomers (enol and imino) and nucleoside isomers (syn) that are unfavoured in aqueous solution. With U substituted for T, we must consider in the case of protein synthesis the pairs that can be formed in this way from the four characteristic bases of nucleic acids and in addition, C-I, U-I, G-I and A-I, since inosine is the first

anticodon residue of many tRNAs⁶. Figure 1 shows complementary pairing schemes (studied by model building) containing I; of these, only C-I utilises the preponderant isomers. The ability of such base oppositions to take up intrahelical arrangements within helices dominated by Watson-Crick pairs has also been experimentally demonstrated, though the actual H-bonding schemes have not been ascertained (ref. 7, and M.D.T., H. Chen and J.R.F., unpublished).

As shown in Fig. 2*a-d*, all the pairs covered by Crick's wobble-pairing schemes make use of the favoured forms of the residues, and the glycosyl bond separation distances are close to the 10.9 Å typical of a complementary pair, for all except A-I, for which it is 12.8 Å. Assuming that the codon constitutes a fixed template, this longer separation distance exaggerates the extent of backbone relocation required of the anticodon chain at the wobble position. In contrast, the amount of chain relocation required for those wobble pairs with the near-normal separation distance appears to be negligible for the short codon-anticodon helical array.

Additional wobble pairs—A-C (and C-A) can be built with the same stereochemistry as their G-U and U-G counterparts (Fig. 3*a,b*) only with a single H bond. They are unlikely to occur since this arrangement would lead to one unfulfilled H-bond

the syn residue lies in a complementary position; but when the template residue is syn, the opposite member takes up a "wobble" position. Thus, the additional pu-pu wobble pairs in Fig. 3 have their complementary inverse pairs; for example, wobble pair A_{syn}-G_{enol,imino} (Fig. 3*c*) has its complementary pair inverse G_{enol,imino}-A_{syn} (Fig. 1*j* of accompanying paper³).

Base pairing in codon-anticodon interaction

From these considerations it is difficult to decide which type of pairing is most relevant to codon-anticodon interaction. Although the complementary non-Watson-Crick pairs are sterically more acceptable, the thermodynamic cost of utilising the unfavourable tautomers or isomers they require could be greater than the cost of a small helix distortion at the wobble position. We have found it possible to choose between these alternatives with the aid of the following three assumptions.

(1) The ribosome limits wobble-pairing schemes to those that minimise backbone distortion and it constrains the message as a template. Studies of coding fidelity with ribosomal mutants⁸ and drugs such as streptomycin^{9,10} indicate that the ribosome does impose steric constraints on codon-anticodon interaction.

(2) The ribosomal cavity for codon-anticodon interaction precludes tautomerism and protonation therein. Complementary

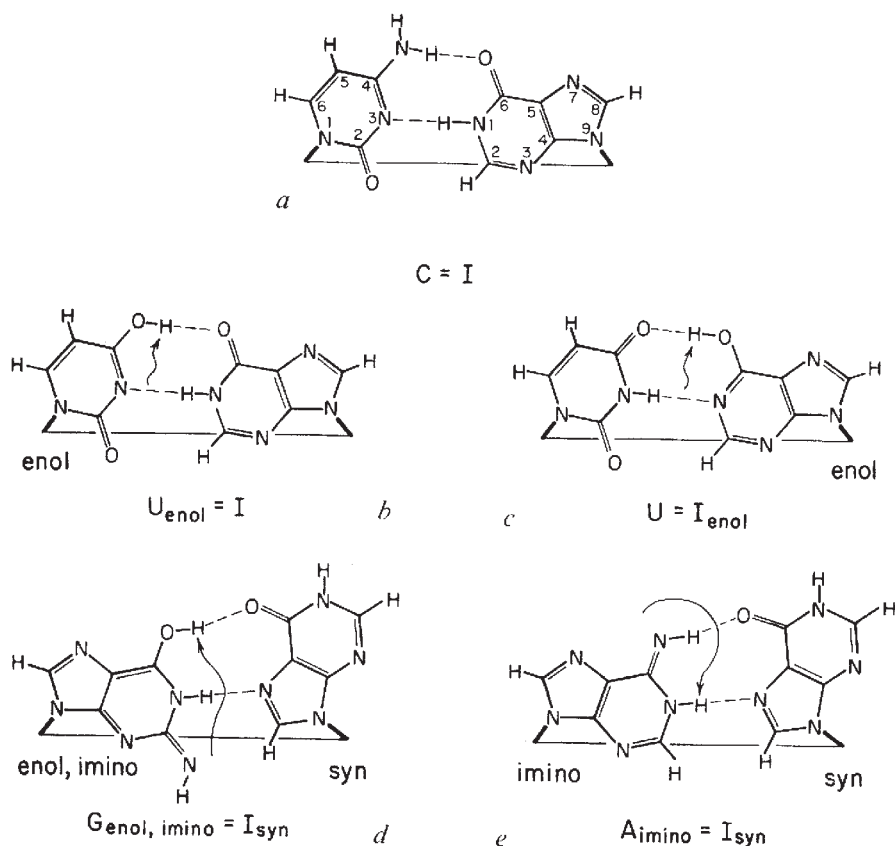


Fig. 1 Complementary base-pairing schemes involving the anticodon I residue. *a-c*, Pyrimidine-purine; *d, e*, purine-purine. The C-I pair (*a*) is in all respects the equivalent of a standard Watson-Crick complementary pair. Other non-Watson-Crick complementary pairs are shown in Fig. 1 of the accompanying paper³ where, however, T is used in place of U. Note that the stereochemistry for the pu-pyr pairs that involve an unfavoured tautomer in either member conforms precisely to that of the Watson-Crick pairs, whereas that for the pu-pu pairs requires a small deviation, $\sim 9^\circ$, in the glycosyl bond angle of the anticodon residue, but no alteration in glycosyl bond separation distance. Wherever shown, arrows indicate the source of a proton that has been transferred in the tautomeric shift from a favoured to an unfavoured isomer.

acceptor site, or two H bonds made possible by protonation of the A member. Additional pu-pu wobble pairs are also conceivable (Fig. 3*c-e*) which make use of combinations of syn isomers and unfavoured tautomers. In particular the A-I pair shown in Fig. 3*e*, which makes use of a syn residue in the codon position has a near-standard glycosyl bond separation distance, instead of the much longer one proposed by Crick (Fig. 2*b*).

When one member of a pu-pu pair has its backbone location fixed as part of a template, an interesting asymmetry exists if one member is syn (Fig. 4). When the template residue is anti,

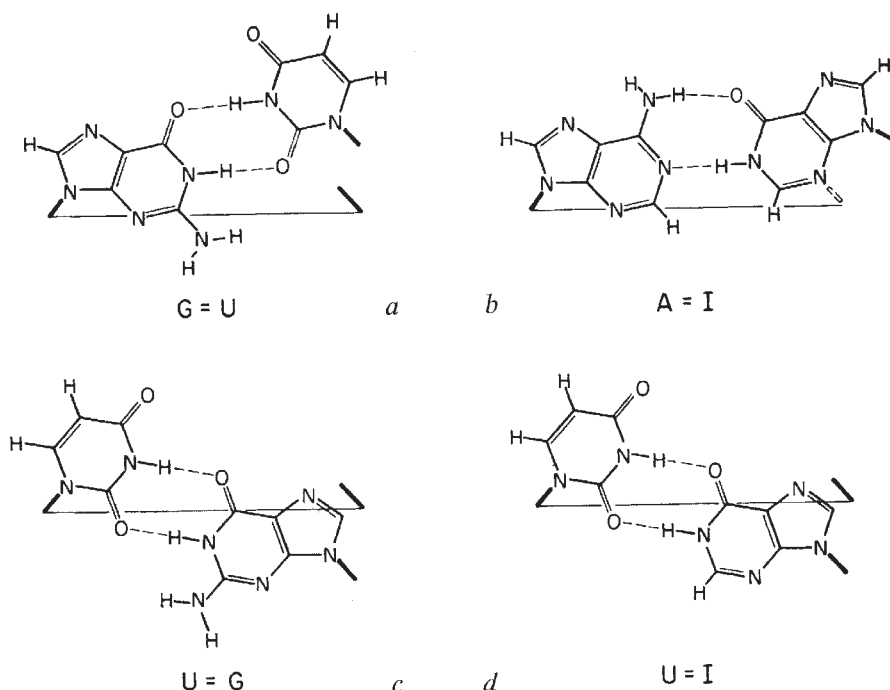
mispairing requires unfavoured tautomers which can occur only by a proton jump by way of water molecules proximal to the donor and acceptor atoms of the base. Binding by the ribosome of both messenger and tRNA must inhibit breathing of the codon-anticodon complex. This alone will preclude tautomerism since water is excluded between paired bases. Moreover, when tRNA and mRNA enter the cavity, bulk water that fills such space in their absence will be displaced, so that in the immediate vicinity of codon-anticodon negotiation, the amount of free water will be greatly reduced. If re-equilibra-

tion to minor tautomers is thereby precluded, miscoding due to complementary mispairs (Fig. 1 of accompanying paper³ and Fig. 1 here) will be kept at a very low level (assumption (3)). Therefore, the specificity at all codon-anticodon positions must depend primarily on the predominant keto and amino forms of the bases.

(3) Tautomeric equilibria nevertheless play a part in codon-anticodon interaction to the extent that the message and tRNA manifest these equilibria in solution. Whereas the ribosome will prevent mismatched oppositions from becoming complementary mispairs (assumption (2)), they must also lock in those unfavoured tautomers that have matched up to form such mispairs (for

occurs at the tRNA wobble position¹⁷ and there are 16 codons with U in the third position. The different geometry for G-U raises the question of whether this pair is also used. Comparison of the code with anticodons established in experiments with sequenced tRNAs (see Table 1 of ref. 17) suggests that G-U may not be required. Thus, Gln, Lys and Gly, with code words that have A or G in the third position (CAA and CAG for Gln, AAA and AAG for Lys, GGA and GGG for Gly), each have two corresponding tRNAs in the same species¹⁸, one with a modified U and another with C in the wobble position. On the other hand, an unusual case has been found in which an ochre suppressor tRNA (UUA) reads the amber codon (UAG) *in vivo*

Fig. 2 Wobble-type or non-complementary H-bonded base pairs proposed by Crick⁴ for codon-anticodon interaction. The codon or template residue is always shown on the left, and the horizontal line from the C1' carbon of the codon residue extends to the location of the C1' carbon of the anticodon residue if the latter were complementary. Note that only keto and amino tautomeric and neutral forms of the bases are used for the pairing schemes proposed by Crick. In each case, the twofold symmetry that relates the C1' carbons of the two residues of a complementary pair is absent. Generally, this leads to only a small amount of backbone displacement because the glycosyl bond separation distance is maintained close to that of a complementary helix. In the case of A-I (b), however, that distance is nearly 2 Å greater, which results in excessive backbone displacement.



example, A_{imino} with C, Fig. 1 of ref. 3). Hence, the low frequencies of minor tautomers that both interactants must contain will be frozen by the ribosome and be expressed. This will give rise to a background *in vivo* level of spontaneous miscoding involving all complementary mispairs since they all involve minor tautomers (G-U, A-C, U-I, G-I, A-I, A-A, A-G, and G-G). Miscoding should therefore occur at all three positions at frequencies that depend on the relative occurrence of the isomers required in the mispairs: for pu-pyr, 10^{-4} to 10^{-5} (tautomeric frequency)¹¹⁻¹⁴; for pu-pu, the latter values are lower, 10^{-5} to 5×10^{-7} because pu-pu pairs involve syn isomers^{15,16} which occur at a frequency of $\sim 10^{-1}$ for G_{syn} and $\sim 5 \times 10^{-2}$ for A_{syn}, as well as unfavoured tautomers.

From these assumptions we can evaluate pairing schemes for those non-Watson-Crick base oppositions that prevail in the wobble position and can then deduce the nature of the mispairs that lead to infidelity. Throughout, base pairs are denoted with the codon residue on the left.

Pairing in the wobble position

U-G, U-I-. Since tautomerism is precluded, Crick's wobble-pairing schemes must prevail. With the codon as template, the glycosyl bond of G or I at the wobble anticodon position will be displaced to the same extent and in the same direction.

G-U-. The deviation of the anticodon residue for wobble G-U, though equal to that for wobble U-G, is opposite in direction of displacement of the anticodon glycosyl bond (Fig. 2a,c). U-I and U-G are required by the code since A never

very effectively¹⁹. In this case at least, G-U has a role even though it is sterically different from U-G.

A-I-. We suggest instead of Crick's "long" wobble pair (Fig. 2b), one in which A in the codon is syn, while I in the anticodon is anti (Fig. 3e). This arrangement, observed in a cocrystal of 9-ethyl-8-bromoadenine and O-ethyl-8-bromohypoxanthine²⁰, uses favoured tautomers and allows a standard glycosyl bond separation distance²¹. With codon as template, however, only wobble geometry is possible (Fig. 4). This arrangement ought to form with acceptable frequency if the 2 kcalorie mol⁻¹ (refs 15, 16) cost of A_{syn} is not increased within the ribosome.

Evidence for an A-I pair in protein synthesis is not definitive. *In vitro* coding data^{22,23} are consistent with its occurrence, but subject to artefacts due to the unnatural levels of divalent cations used²⁴. Moreover, it seems that there are two tRNAs with I in their anticodons (one for Val and one for Ser) that are not needed for interaction with codons (GUA and UCA, respectively) that have A in the third position¹⁷ since isoacceptor tRNAs exist with U in place of I. Because these isoacceptors have not yet been demonstrated in the same species, however, their bearing on the question is uncertain. Conceivably, a tRNA with I instead of U in the anticodon is required to prevent the ambiguous reading of the Met codon, AUG, by a tRNA^{11c} that normally reads AUA. It is now apparent^{25,26}, however, that ambiguous recognition of G instead of A is avoided by replacing U with s²U. In any case, if A-I occurs *in vivo*, then the A_{syn}-I wobble arrangement would seem to be more favourable than Crick's⁴.

A-G-. Although this pair occurs in long Watson-Crick helices (M. D. T., H. Chen and J. R. F., unpublished), the code forbids it at the wobble position. Crick suggested⁴ that the A-G counterpart to the A-I wobble pair does not occur because the amino group of G cannot H bond even to water. If A-I has a role in protein synthesis, this presumed constraint would be equally relevant to the A_{syn} -G counterpart to the proposed A_{syn} -I pair. Moreover, it seems that a 2-amino hydrogen of G eclipses the C8 hydrogen of A_{syn} . In either case, a tautomeric shift (2-amino $\rightarrow$ 6-oxygen) could remove this constraint. As noted, however, such shifts are precluded within the ribosome. If instead, A-I does not occur *in vivo*, then both A-I and A-G may be excluded by the same ribosomal constraints that prevent A from either becoming syn or pairing with a long glycosyl bond separation distance.

A-C, G-G, G-A, C-A-. These pairs also occur in long Watson-Crick helices (M.D.T., H. Chen and J.R.F., unpublished), even though they are prohibited by the code²⁷.

the U-G, G-U and U-I pairs there (because these pairs could not then occur with the high frequency required), while the original H-bonding schemes of the wobble hypothesis are correct except for the case of A-I. A more reasonable wobble-pairing arrangement is proposed for A-I that is consistent with the absence of the analogous A-G pair.

Mispairing in codon-anticodon interaction

We now evaluate the role of non-Watson-Crick pairs as a source of infidelity in protein synthesis. It is evident from the prohibition of wobble pairs at the first two codon positions that the ribosome effectively screens out non-complementary pairs from those positions. In normal *in vivo* conditions, however, there ought to be a background level of miscoding that reflects the frequency of residue isomers on both message and tRNA before they enter the ribosome (assumption (3)), that can give

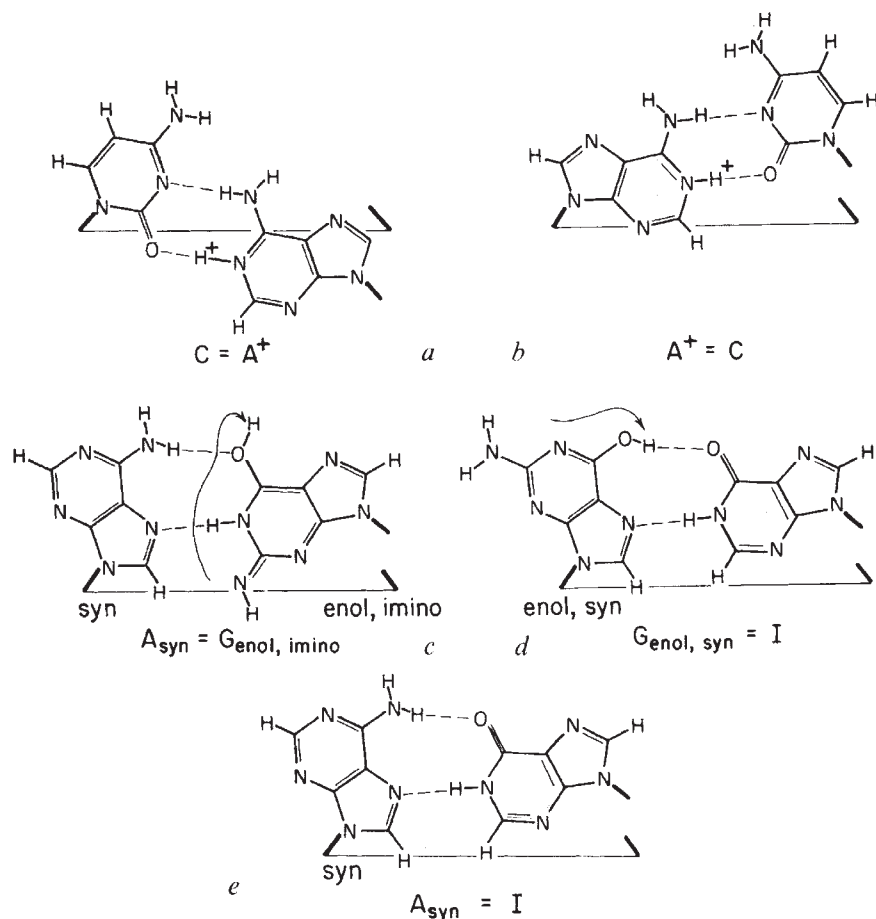


Fig. 3 Additional wobble-type base pairs for consideration in codon-anticodon interaction. A-C and C-A (a,b), which do not normally occur in codon-anticodon interaction, can only make this type of pair with two H bonds when one of its members is protonated. Among the pu-pu pairs, all involve one member in the syn configuration, but A_{syn} -I (e) is unique in not requiring an unfavourable tautomer. The requirement for an unfavoured tautomeric shift in the case of A_{syn} - $G_{enol, imino}$ (c) is not to enable the H bonding itself, but to remove an otherwise interfering interaction of the 2-amino group of G with the C8 hydrogen of A.

Presumably, A-C and G-G do not serve at the wobble position because their pairing schemes require minor tautomers in high frequency, and G-A and C-A are irrelevant since A never occurs in the wobble position of tRNA⁶.

U-U, C-C, U-C, C-U-. These do not occur in long Watson-Crick helices (J. Balcerski, M.D.T. and J.R.F., unpublished), and are forbidden by the code. Their occurrence would require a large backbone distortion that would enhance electrostatic repulsion of the phosphates brought closer together. The exception of U-U⁵-oxacetate pairing²⁸ must be due to some unique effect of the substituent on C5 of the pyrimidine on the tRNA.

We conclude that schemes for pairing at the wobble position that require minor tautomers of bases^{3,29} cannot account for

rise to complementary mispairs at all three codon positions. Mispairing at the wobble position could possibly also arise from the additional wobble pairs proposed in Fig. 3, since the ribosomal constraints must be relaxed somewhat to allow "normal" wobble at that position. Higher levels of miscoding due to mutations that reduce the ribosomal constraints or to drugs with similar consequences should be superimposed on this natural level of infidelity. Still higher levels of miscoding should occur *in vitro* when unnatural concentrations of cations or other artefactual conditions are used that overcome the specificity of the codon-anticodon interaction. Miscoding in this instance could involve an even wider range of non-Watson-Crick base oppositions, particularly if only two base pairs have to be stabilised.

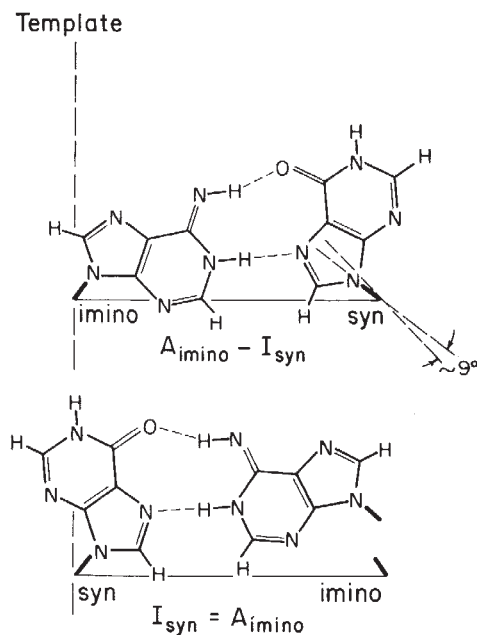


Fig. 4 The steric relationship between a complementary pu-pu pair, when one residue has its locus fixed as part of a template, and either member is in the syn configuration. The particular example shown is that of an A-I pair with A as template (top) and I as template (bottom). Whereas the former meets complementary base-pair glycosyl bond separation distance without a significant dislocation of the backbone segment to which the non-template base is linked, wobble-type dislocation results when the syn residue is on the template.

Evaluation of the mispairing hypothesis

In vivo measurements of suppression of polar mutations in structural genes for β -galactosidase³⁰ and ornithine transcarbamylase⁸ and of the frequency of incorrect insertion of amino acids into rabbit haemoglobin and chick ovalbumin^{31,32} lead to values of infidelity as high as 10^{-4} per amino acid site. This is consistent with what is expected from the frequency of the most favoured complementary mispairs, that is, those requiring only unfavoured tautomers (10^{-4} – 10^{-6}). Thus, the *in vivo* infidelity is of a level that might be expected from the frequency of the minor tautomers in both message and tRNA before their interaction within the ribosome.

The *in vivo* level of infidelity is the sum of errors in several steps. Errors due to complementary mispairs will contribute a maximum frequency of 5×10^{-5} per residue in both transcription and codon-anticodon interaction. This is equivalent to an infidelity frequency of 3×10^{-4} per amino acid site, ignoring degeneracy of the code, which would lower this value. The indications are³³ that amino acid activation and aminoacylation together contribute another factor of 10^{-4} , for an overall error level of 4×10^{-4} , which is consistent with that measured *in vivo*. Although kinetic proofreading³⁴ of the codon-anticodon interaction may be necessary to screen out errors from such effects as two-base pair interactions, there is no obvious basis and no apparent need for proofreading of infidelity caused by complementary mispairs. This is because the mispairs are frozen within the ribosome and the level of infidelity to which they give rise is not greater than that contributed by the other steps of protein synthesis. Indeed, energy used for proofreading at this stage would seem wasteful.

If complementary mispairing is a source of errors in codon-anticodon interaction, there should be a defined pattern of miscoding which involves all complementary mispairs, that is, U-G, G-U, A-C, C-G and A-G at all codon positions, A-A, G-A and C-A at only the first and second positions (since

tRNAs never contain A in the wobble position), and G-I at only the third (since I only occurs at the wobble position). While pyr-pyr pairs should not occur at any codon position, additional A-C, A-G and G-I wobble-type pairs (Fig. 3) may possibly occur at the third position. The only *in vivo* coding data³⁰ confirms the occurrence of G-U, and A-C in both the second and third codon positions, with the frequency of background suppression due to A-C occurring at the 0.01% level, which is consistent with the frequency of required tautomers. A higher value was observed for G-U, however; reasons for this are not apparent.

In vitro studies of codon-anticodon interaction do not provide a basis for evaluating the mispairing hypothesis. The errors occur at 10–100-fold or greater frequency than *in vivo*³⁴, presumably because of artefactual stabilisation of a variety of "nonspecific" interactions.

This investigation of base pairing in codon-anticodon interaction provides a basis for refinement of the wobble hypothesis and a chemical basis for infidelity in the process. Although our approach follows that which we used to investigate base pairing in nucleic acid biosynthesis³, the present analysis is less satisfying because of the paucity of data against which to evaluate our hypothesis on base pairing in protein synthesis, but the concepts presented provide a basis for experimental focus.

This study has led us to consider the role of the ribosome in protein synthesis. It seems that the specificity of codon-anticodon interaction derives as much from the ribosomal constraints as from the limited degree of specificity that can come from the base-pairing interactions. The development of such constraints, which assure biologically acceptable levels of fidelity and efficiency of protein synthesis, may have been crucial during evolution of the ribosome.

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Formation of stable crystalline enzyme-substrate intermediates at sub-zero temperatures

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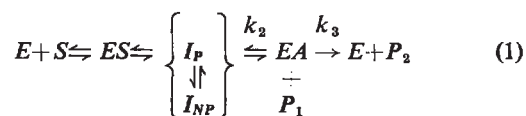
Sub-zero temperatures can be used to trap intermediates in enzyme-catalysed reactions using suitable cryosolvents. The feasibility of obtaining such intermediates in the crystalline state for X-ray diffraction studies has been demonstrated with several proteases, using specific substrates and optimal pH.

Two problems in the study of enzyme mechanisms are the rapidity of the reaction and the low concentration of intermediates present in steady-state conditions. These cause technical difficulties in the determination of events occurring during the dynamic processes of catalysis. The existence and structure of intermediates in the catalytic reaction pathway must be known before the mechanism can be ascertained, and the great catalytic efficiency of enzymes can be explained. X-ray crystallography is one of few techniques that can yield structural data about such intermediates at the desired resolution. Unfortunately X-ray diffraction is limited in that it is essentially a static procedure and requires stable, crystalline compounds. Recent developments, however, suggest that this might be overcome at sub-zero temperatures.

It has been known for some time that many enzymes are catalytically active in aqueous organic solvent systems at sub-zero temperatures¹⁻⁶. We have been exploiting different free energies and enthalpies of activation for the different steps in the overall enzyme-catalysed reaction to accumulate intermediates at very low temperatures. The basis of this approach is that when the reaction is initiated by mixing enzyme and substrate at a very low temperature the rates of intermediate transformations with sufficiently high activation energies will become negligible. Consequently, turnover will not occur and intermediates will accumulate. Thus instead of steady-state turnover, a series of reaction steps will be seen, each corresponding to the transformation of one intermediate into

another as the temperature increases. Eventually a temperature will be reached that is sufficiently high for turnover to occur. As long as the temperature is sufficiently low to avoid turnover, the enzyme will be trapped in the form of an intermediate or intermediates. The method is discussed in more detail elsewhere⁹. For several enzymes it has been possible to demonstrate the accumulation of intermediates found in the reaction with kinetically specific (good) substrates at optimal pH by this technique^{3,6,10-15}. In these studies dissolved enzyme and various spectral techniques were used to detect and characterise the trapped intermediate.

We have investigated the feasibility of using sub-zero temperatures to obtain the trapped enzyme-substrate intermediates in crystalline form and sufficiently stable for high resolution X-ray diffraction studies. The intermediates used were acyl-enzymes formed from serine proteases. The reaction scheme for these enzymes can be represented by equation (1), in which *ES* is the initial non-covalent Michaelis complex, *I_P* and *I_{NP}*



represent possible subsequent productive and non-productive intermediates respectively, *EA* is the acyl-enzyme intermediate and *k₂* and *k₃* are the rate constants for acylation and deacylation.

The serine proteases were chosen for several reasons. We have shown that sub-zero temperatures and 65% aqueous dimethyl sulphoxide or 70% aqueous methanol have no adverse effects on the structural and catalytic properties of chymotrypsin and trypsin^{6,7}. We have also shown that essentially stoichiometric amounts of acyl-chymotrypsin, formed from specific *p*-nitrophenyl esters, can be isolated at sub-zero temperatures⁶. For specific ester substrates of chymotrypsin the acylation reactions can be orders of magnitude faster than

Table 1 Acylation and deacylation rates and acyl-enzyme concentrations for dissolved enzymes

Enzyme	Substrate	Solvent	pH*	Temperature (°C)	[S] ₀ (M × 10 ³)	[E] ₀ § (M × 10 ⁶)	EA† (%)	Acylation <i>k</i> _{obs} (s ⁻¹)	Deacylation‡ <i>v</i> ₁ (mol s ⁻¹)
Elastase	ZAP	70% MeOH	7.2	-43.4	3.2	13.4	82	1.1 × 10 ⁻³	
Elastase	ZAP	70% MeOH	5.7	-50.1	0.75	5.1	92	3.2 × 10 ⁻⁴	
Elastase	ZAP	70% MeOH	7.2	-37.0	1.5	5.6	79	1.8 × 10 ⁻³	1.2 × 10 ⁻¹⁰
Elastase	AcAla ₃ Me	70% MeOH	7.2	-52.7	1.9	1.6	47	2.3 × 10 ⁻⁴	
α-Chymotrypsin	AcTrpP	60% DMSO	5.7	-41.5	1.0	6.6	86	5.0 × 10 ⁻⁴	6.2 × 10 ⁻¹²
δ-Chymotrypsin	AcTrpP	52% DMSO	5.7	-35.3	1.4	7.6	61	1.3 × 10 ⁻³	4.6 × 10 ⁻¹¹
γ-Chymotrypsin	AcTrpP	52% DMSO	5.7	-41.2	1.5	17.0	44	7.6 × 10 ⁻⁴	1.2 × 10 ⁻⁹
γ-Chymotrypsin	AcTrpP	70% MeOH	7.0	-72.9	0.7	48.0	83	1.3 × 10 ⁻⁴	9.1 × 10 ⁻¹⁰
γ-Chymotrypsin	AcTrpP	70% MeOH	5.1	-43.6	4.1	6.1	82	6.6 × 10 ⁻⁴	2.4 × 10 ⁻¹⁰
γ-Chymotrypsin	AcTrpP	70% MeOH	6.7	-56.1	1.2	6.4	100	3.6 × 10 ⁻⁴	9.6 × 10 ⁻¹¹
Trypsin	ZLysP	65% DMSO	7.7	-32.6	1.0	28.0	44	3.2 × 10 ⁻⁴	4.5 × 10 ⁻¹⁰

Substrate abbreviations: Z, N-carbobenzoxy; A, Ala, L-alanine; P, *p*-nitrophenyl ester; Ac, acetyl; Me, methyl; Trp, L-tryptophan; Lys, L-lysine. Solvents were 70% aqueous methanol (acetate buffer); 60 or 65% aqueous dimethyl sulphoxide (acetate) (DMSO); and 52% aqueous dimethyl sulphoxide, saturated with ammonium acetate at -80°C (52% DMSO). Apparent protonic activity, pH*, of the aqueous organic solvents was measured at 25°C and extrapolated to sub-zero temperatures using standard curves⁴¹.

*Active-site normalities of the enzyme preparations were determined by standard procedures³¹.

†Acyl-enzyme concentration expressed as fraction of total enzyme concentration. Estimated error ±15%.

‡Observed initial rate of deacylation (turnover) (see text).

Table 2 Rates of acylation and deacylation, and active-site occupancies for crystalline enzymes

Enzyme	Substrate**	Crystal† size (μm)	Solvent**	pH*	Temperature ($^{\circ}\text{C}$)	[S] ($\text{M} \times 10^3$)	Solubility (M)	EA‡ (%)	Acylation k_{obs} (s^{-1})	Deacylation k_{obs} (s^{-1})
Elastase	ZAP	320	70% MeOH	7.2	-46.1	1.0	2.1×10^{-8}	86	2.7×10^{-5}	
Elastase	ZAP	1,200	70% MeOH	5.1	-47.8	0.6		≥ 55	7.8×10^{-6}	0
Elastase	AcAla ₃ Me	80	70% MeOH	7.2	-54.5	2.8		≥ 86	1.35×10^{-5}	0
α -Chymotrypsin	AcTrpP	90	52% DMSO	6.1	-58.2	8.6	1.4×10^{-5}	3	$\geq 4.1 \times 10^{-5}$	0
δ -Chymotrypsin	AcTrpP	Amorphous	52% DMSO	6.1	-58.2	7.6	6.1×10^{-6}	82	$\geq 4.1 \times 10^{-5}$	0
γ -Chymotrypsin	AcTrpP	25	52% DMSO	5.8	-49.4	7.0	7.0×10^{-6}	≥ 61	$\geq 5.4 \times 10^{-5}$	0
Trypsin	ZLysP	70	52% DMSO	6.1	-58.2	9.8	2.0×10^{-5}	66	$\geq 4.1 \times 10^{-5}$	0

**See footnotes to Table 1.

†Longest dimension.

‡The reported values are minimum ones. Estimated errors are $\pm 25\%$.§This reaction appeared biphasic, the first part of the reaction with $k_{\text{obs}} = 2.4 \times 10^{-4} \text{ s}^{-1}$.

deacylation at temperatures below -40°C in 65% aqueous dimethyl sulphoxide^{6,7,10,11}. We have also detected and accumulated intermediates in addition to the acyl-enzyme in the reactions of chymotrypsin, elastase and trypsin with specific substrates^{6,7,10,11}. Since our studies suggest that these intermediates are present, although not readily detected, when the reactions are carried out in "normal" conditions, knowledge of their structure is desirable¹⁰.

Crystals of chymotrypsin and elastase have been shown to be catalytically active in aqueous solution at above-zero temperatures¹⁶⁻²³. Preliminary studies on the effects of crystal size on the reaction rate and substrate diffusion in enzyme crystals have shown that catalytic rates and accessible active-site concentrations depend on crystal size²⁴⁻²⁶. Crystallographic studies have been carried out on acyl-enzymes formed by the reaction of nonspecific substrates with chymotrypsin²³, and on enzyme-substrate complexes formed by reaction of serine proteases with virtual substrates²², peptide competitive inhibitors^{17,18}, peptide chloromethyl ketones¹⁶ and protein inhibitors²⁷. These investigations have greatly furthered the understanding of the mechanisms of action of these enzymes. But the fact that the derivatives used were sufficiently stable for X-ray diffraction implies that the relative positions of the enzymes' catalytic groups and the substrate were not optimal.

Petsko^{28,29} has shown that X-ray diffraction studies of enzymes at sub-zero temperatures using aqueous-organic solvent systems have advantages, such as greatly decreased radiation damage. If X-ray diffraction techniques are to be applied to crystalline, trapped enzyme-substrate intermediates the following requirements must be met. At least 25 per cent of the enzyme must be in the form of the intermediate in order to attain sufficient intensity in electron density difference maps. Only free enzyme or the desired intermediate should be present. Mixtures of more than one type of intermediate are undesirable, and the crystalline intermediate, once formed, must be stable for a sufficiently long period for diffraction data to be collected, probably a minimum of 1-4 d for high resolution studies. We have shown, using sub-zero temperatures, that intermediates such as acyl- and glycosyl-enzymes satisfy the latter two requirements^{6-8,10-13}. This and the accompanying article³⁰ confirm the validity of the cryoenzymological approach for obtaining detailed structural information on enzyme-substrate intermediates.

Acyl-A enzyme formulation in solution

p-Nitrophenyl ester substrates are well suited for studies of acyl-enzyme formation and breakdown in the serine proteases. The chromophoric nitrophenol moiety provides a simple means of monitoring the reaction. The smaller rate constant and larger energy of activation for the deacylation step mean that at appropriately low temperatures not only is the rate of acylation much faster than that of deacylation but also that the latter can be essentially zero.

The acylation and deacylation reactions of the following enzyme-substrate combinations were studied in the range -20 to -70°C using aqueous dimethyl sulphoxide and aqueous

methanol cryosolvents: α -, δ -, and γ -chymotrypsin with *N*-acetyl-L-tryptophan *p*-nitrophenyl ester (AcTrpP), trypsin with *N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester, and elastase with *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester (ZAP) and *N*-acetyl-L-alanyl-L-alanyl-L-alanine methyl ester.

Rates of acylation and deacylation (turnover) were determined for the *p*-nitrophenyl ester substrates by monitoring the release of *p*-nitrophenol at 340 or 350 nm. The concentration of acyl-enzyme formed was estimated from the amount of *p*-nitrophenol released in the first-order acylation reaction. For dissolved enzyme experiments, the enzyme stock solution was diluted 1:4 with cryosolvent at 0°C and then added to the substrate solution at the desired sub-zero temperature.

For each enzyme, the rates of acyl-enzyme formation and breakdown, and the maximum concentration of acyl-enzyme formed were determined in various conditions. Some representative results of such experiments with dissolved enzymes are given in Table 1. In each case the acylation was first order and very much faster than deacylation. Deacylation was much too slow for the reactions to be followed to completion in a convenient time. In Table 1 the deacylation rates are reported in the form of the observed initial velocities (v_i). The substrate concentrations used were of the order of K_m ; hence, dividing v_i by the acyl-enzyme concentration gives an approximate value of the microscopic deacylation rate constant, k_3 in equation (1).

For each enzyme there are conditions in which the rate of deacylation is sufficiently slow that negligible amounts of acyl-enzyme would be lost during the time necessary for the collection of diffraction data. For example, for elastase in 70% aqueous methanol at pH* 5.7 the amount of dissolved *N*-carbobenzoxy-L-alanyl-elastase which will hydrolyse in 24 h is 0.4% at -70°C , 2.2% at -60°C , and 11.0% at -50°C . Acyl-enzyme concentrations approximately stoichiometric with the enzyme concentrations were only obtained at temperatures sufficiently low that no turnover was observed.

In conditions in which turnover occurs, the amount of enzyme converted into acyl-enzyme is predicted to be proportional to the substrate concentration unless it is much greater than K_m (ref. 31). This was observed to be the case. For example, with elastase and ZAP the fraction of total enzyme in the form of acyl-enzyme was a function of temperature; at increasingly higher temperatures, as the deacylation rate increased, the proportion of acyl-enzyme decreased. Consequently to obtain the maximum concentration of acyl-enzyme, it is essential to adjust pH* and temperature such that deacylation is negligible.

Experiments with dissolved α -, δ -, and γ -chymotrypsin indicate that approximately stoichiometric concentrations of acyl-enzyme can be obtained in the -50°C to -70°C range, using 65% aqueous dimethyl sulphoxide at pH* 5-8. Since both acylation and deacylation have similar pH dependencies, the relative ratio of rates of acylation to deacylation is essentially pH independent. In the conditions used, the rates of acylation were several orders of magnitude greater than that for deacylation. The rates of deacylation, especially at temperatures below -50°C and at low pH*, were so slow that the acyl-enzymes could be made stable for a time scale of weeks or longer.

Because aqueous dimethyl sulphoxide cryosolvents are much

more viscous, they are less desirable than the corresponding methanol solvents³². The nucleophilicity of methanol towards acyl-chymotrypsins, however, is much greater than that of water³³, which suggested that in experiments at sub-zero temperatures deacylation would be much faster with aqueous methanol than with aqueous dimethyl sulphoxide for the same temperature. As Table 1 shows, with γ -chymotrypsin and AcTrpP the deacylation rate was about 15 times greater in the methanol solvent. This value is in good agreement with that calculated from the data of Bender *et al.*³³ obtained at 25 °C.

Crystalline acyl-enzyme formation

As preliminary attempts to crystallise the trapped acyl-enzymes at very low temperatures were unsuccessful, we started with the enzymes already in the crystalline state. Crystals of the enzymes were prepared as described before^{34,35}, except that γ -chymotrypsin was crystallised using 40% saturated ammonium acetate, pH 5.6, rather than ammonium sulphate³⁴. Crystals were grown in small capillary tubes, closed with dialysis membrane³⁶. For most experiments with crystalline enzyme the procedure was as follows. One or more crystal(s) of the enzyme was added to the cryosolvent, either directly at 0 °C, or stepwise²⁹. The crystal(s) was then added to the substrate solution at the desired temperature or vice versa. The reaction was either monitored directly, or samples were removed and analysed spectrophotometrically for *p*-nitrophenol. For experiments with elastase and the peptide ester substrate, acylation was followed by assaying for free elastase using ZAP at -40 °C. The solubilities of crystalline enzymes were determined by assaying samples from control experiments in which the substrate had been omitted. All experiments were carried out in conditions of excess substrate.

The precision of the values of the active-site occupancies of the crystals is not very high because of the difficulty of ascertaining the concentration of active sites in the crystal. The most accurate values were obtained when the crystalline acyl-enzyme deacylated at higher temperatures and was then dissolved in a suitable solvent for determination of total protein concentration. In conjunction with active-site normality titrations of the same batch of enzyme, this facilitated estimation of the active-site concentration in the crystal.

The data given in Table 2 are for experiments in which the enzymes were added in the crystalline state. With the exception of α -chymotrypsin, sufficient active-site occupancy was achieved for satisfactory X-ray diffraction studies. Crystalline and dissolved acyl-enzymes were shown to possess full potential catalytic activity when their temperatures were raised to 0 °C and the rates of turnover were measured.

Ammonium acetate was added to reduce the solubility of chymotrypsin and trypsin in 65% aqueous dimethyl sulphoxide. Experiments with dissolved enzyme indicated that the catalytic reaction was essentially the same in both 65% aqueous dimethyl sulphoxide ($\mu = 0.1$ M) and in 52% aqueous dimethyl sulphoxide, saturated (at -80 °C) with ammonium acetate, at the same temperature and pH*.

α -Chymotrypsin crystallises in a dimeric form in which the active site is blocked by the adjacent molecule. This packing geometry hinders access by any but the smallest of substrates or inhibitors^{16,38}. Our observation that only 3% of the active sites of crystalline α -chymotrypsin was occupied using AcTrpP compared with 20 times as much with γ -chymotrypsin in similar conditions, not only is in accord with expectations but serves as a valuable control. Active-site occupancies with crystalline α -chymotrypsin of 15–20% at 25 °C have been claimed with similar substrates¹⁹. Attempts to crystallise δ -chymotrypsin have been unsuccessful³⁷, and so it was used in the amorphous state. The high active-site occupancy observed with this physical form of the enzyme is in accord with predictions that in very small crystals diffusion into the crystal is not limiting^{24–26}. The active-site occupancy obtained with γ -chymotrypsin crystals is of the desired order for X-ray diffraction experiments.

As might be expected the results with trypsin are very similar to those for γ -chymotrypsin. For the crystalline enzyme the active-site occupancy and acylation-deacylation rate ratio indicate the suitability of trypsin for diffraction studies using aqueous dimethyl sulphoxide and temperatures below -50 °C. The explanation for the lower active-site occupancies with γ -chymotrypsin and trypsin is probably due to errors in determining the concentration of the active enzyme in the crystals.

For elastase a polypeptide ester substrate as well as a *p*-nitrophenyl ester was used. Both the molecular size and the kinetic parameters, k_{cat} and K_m , are very similar for ZAP and *N*-Ac-Ala-Ala-Ala methyl ester^{39,40}. We have also found that non-covalent enzyme-substrate intermediates involving elastase and *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide can be accumulated in a similar fashion to the acyl-enzyme (unpublished results). Most of the elastase experiments were carried out using aqueous methanol. The solubility of elastase was found to be much less than that of trypsin and chymotrypsin in the cryosolvents used. The results from experiments using crystalline elastase indicated that the active site occupancy for the peptide substrate was at least equal to or better than that for the *p*-nitrophenyl ester. The acylation reaction for the latter substrate with the largest enzyme crystals seemed biphasic (Table 2). The data obtained show that substantial concentrations of crystalline acyl-elastases can be obtained in 70% aqueous methanol at the pH-optimum and that at temperatures below -60 °C the acyl-enzymes will be stable for several days. Good agreement in the observed rates of acylation and deacylation for crystalline elastase with ZAP was found in the results reported here and those obtained in the diffraction experiments³⁰.

Previous studies have indicated that at 25 °C the rates of reactions catalysed by crystalline enzymes range from 100 to 0.3% of the rate with the enzyme in solution^{24–26}. At sub-zero temperatures we observed substantially slower acylation when the crystalline enzymes were used than in the corresponding reaction with dissolved enzyme. For example, acylation was approximately 20 times slower with the crystalline enzyme for elastase and about 15 times slower for γ -chymotrypsin. As Table 2 shows, no deacylation was observed with the crystalline acyl-enzymes over 2–4-d periods at temperatures below -40 °C. This observation is in contradiction to the predicted deacylation rates based on the experiments using the dissolved acyl-enzymes (Table 1) and suggests that the intrinsic rate of deacylation is slowed down in the crystal by an amount at least comparable with that of the acylation. One interpretation of this result—that the crystal lattice forces are responsible and perhaps hinder the deacylation by impeding a conformational change—is supported by the observation that in crystalline trimethyl-acetyl-chymotrypsin (ambient temperature, aqueous buffer) the rate of deacylation is 50–100 times slower than in solution, and that the crystals shatter after 5–10% deacylation (F. J. Kézdy, personal communication). These observations are not in accord with the report that indoleacryloyl-chymotrypsin had identical rates of hydrolysis when in solution or crystalline phase⁴².

In conclusion, the results of this investigation show that temperatures in the -50 °C to -70 °C range (depending on the pH*), in conjunction with suitable cryosolvents, can be used to accumulate essentially stoichiometric concentrations of dissolved acyl-enzymes from serine proteases and specific substrates. Furthermore, substantial concentrations of acyl-enzymes in the crystalline state can be obtained in a similar manner. These experiments can be done at pH values corresponding to maximum activity. The accumulated acyl-enzymes (dissolved or crystalline) are stable for periods of days to months. By the choice of appropriate pH* and temperature the active-site occupancies and negligible deacylation rates necessary for X-ray diffraction studies can be attained. Confirmation of the results and predictions of this study are given in the accompanying article³⁰ concerning crystallographic studies of an acyl-elastase.

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Crystal structure of elastase-substrate complex at -55°C

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The structure of a specific acyl-enzyme intermediate in the elastase-catalysed hydrolysis of N-carbobenzoyl-L-alanyl-p-nitrophenol ester has been determined by X-ray diffraction at 3.5 Å resolution. The acyl-enzyme was stabilised by cooling the crystal to -55°C during substrate addition and data collection.

THE structure of a specific acyl-enzyme intermediate in the catalytic reaction of a serine protease has been determined by the X-ray diffraction at sub-zero temperatures. The use of a fluid cryoprotective solvent methanol-water, allowed addition of the substrate to the crystalline enzyme at -55°C . At this temperature an acyl-enzyme formed within 1 d but was stable for more than a week. This work demonstrates the potential of low temperature protein crystallography for directly determining the details of the interactions of enzymes with their actual substrates.

Cryoenzymology

Protein crystallography is the most powerful technique for observing the interactions of enzymes with small molecules at atomic resolution. Since protein crystals contain intermolecular channels filled with the liquid of crystallisation, it is possible to diffuse small molecules into the crystals. There they can bind to the protein molecules if their binding sites are not blocked by molecule-molecule contacts. The crystal structure of the complex can then be determined by the usual techniques. The interactions of inhibitors and pseudo-substrates with crystalline enzymes have been

studied in this way^{1,2}. Until now, however, it has not been possible to use this approach to study the binding of an actual substrate, because collection of a full set of three-dimensional X-ray diffraction data at high resolution takes at least several days, while the lifetime of a productive enzyme-substrate complex, even in the crystalline state, is several seconds or less. Thus, averaged over the time of data collection, the observed structure will contain only a negligible amount of enzyme-substrate complex.

The structure of a productive enzyme-substrate complex or an intermediate on the catalytic pathway could be determined crystallographically if the rate of enzyme catalysis could be reduced considerably. Douzou has pointed out that this can be done by lowering the temperature³. The Arrhenius equation relating reaction rates to temperature predicts that an enzymatic reaction having an energy of activation of $-15\text{ kcalorie mol}^{-1}$ will be slowed by a factor of 10^9 on cooling from room temperature to -100°C . At this low temperature an enzyme-substrate complex with a lifetime of 10^{-3} s at $+25^{\circ}\text{C}$ would be stable for 1.2 d. Douzou and his colleagues have made use of this effect to study some enzyme-substrate interactions in solution spectroscopically⁴ and have also determined the physicochemical properties of many aqueous organic mixed solvent systems⁵. These solvents remain fluid at very low temperatures and, if their physicochemical parameters are properly adjusted, do not denature enzymes nor alter the rate-determining reaction step^{6,7}.

Recently Fink and his coworkers made a detailed study of the reactions of several serine proteases in both solution and crystalline states with ester and amide substrates in aqueous organic solvents at sub-zero temperatures^{8,9}. They concluded that in carefully controlled conditions the enzymes would function normally in this bizarre environment but

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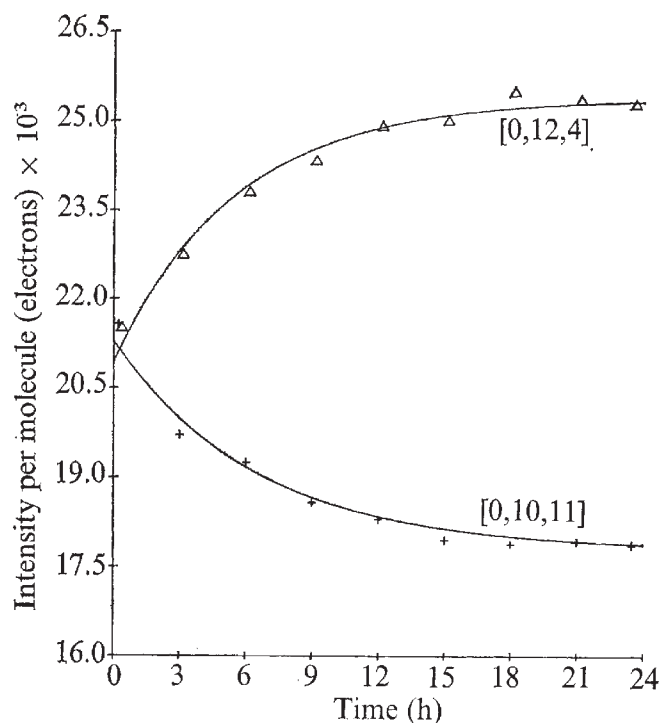


Fig. 1 Plots of the changes in intensity with time of two high-resolution $0kl$ reflections of elastase as substrate is added. The solid curves are calculated assuming a first-order rate constant of $5.4 \times 10^{-5} \text{ s}^{-1}$.

their rates were so reduced as to make direct observation of short lived proteolysis intermediates feasible. Fink has also determined conditions in which sufficiently high concentrations of such intermediates can be accumulated in crystals and has characterised the species formed¹¹. These studies are invaluable for planning and interpreting X-ray diffraction experiments at low temperatures.

We have extended the techniques of cryoenzymology to protein crystallography by cooling protein crystals immersed in fluid mixed solvents, and then flowing in substrate at low temperature. In this paper we report the results of such an experiment with a specific synthetic substrate of porcine pancreatic elastase at -55°C in 70% methanol–30% water $p\text{H}^* 5.2$ ($p\text{H}^*$ denotes protonic activity in mixed solvents at the specified temperatures^{8,11,13}).

Cryoprotection of elastase crystals

We chose a serine protease for our first experiments because there is a large body of kinetic and structural data available for this class of enzymes, but many details of their mode of action are still disputed⁸. They are also attractive because the rate-determining step in the reaction differs for different types of substrate. This offers the possibility of trapping several different intermediates crystallographically at low temperatures¹⁰. We chose elastase as the particular crystalline enzyme to study because of its low solubility and high stability in alcohol–water mixtures¹¹ and the very favourable molecular packing in its crystals. The active site of the enzyme faces a solvent-filled interstice 18 Å in diameter, allowing the binding of large peptide substrates¹².

Elastase crystals are grown from sodium sulphate–sodium acetate solutions at low ionic strength. They are normally stabilised in 1.2 M sodium sulphate, 0.01 M acetate ($p\text{H} 5.0$) and it was in this salt-rich mother liquor that the room temperature crystal structure was solved by Watson and his colleagues¹². To cool the elastase crystals to temperatures in the -50°C range (which we knew to be sufficiently low to stabilise an enzyme–substrate complex from the work of Fink and his colleagues¹¹), it was first necessary to find a

replacement mother liquor which would not freeze¹³.

We found that elastase crystals are stable in a wide variety of mixed solvents, provided certain procedures are followed. The crystals cannot be transferred directly from 1.2 M sodium sulphate to alcohol–water mixtures, because of the low solubility of sodium sulphate in alcohol. The first step of the transfer is therefore to place the crystals in 0.01 M sodium acetate ($p\text{H} 5.0$). In this salt-free liquid they are stable for weeks at 4°C . Transfer cannot be made directly to solvents of high alcohol concentration, as this cracks and partially disorders the crystals. Transfer can safely be made, however, to solvents of low alcohol concentration, and the percentage of alcohol may be increased to any desired level provided the gradual addition of alcohol is coupled with a gradual reduction in temperature¹³. Alcohol concentration and temperature are varied so as to keep the dielectric constant of the solvent as near as possible to the value for pure water^{4,13}. It is important at each step to vary the $p\text{H}$ of the aqueous buffered component to keep the protonic activity of the solvent constant^{4,13}. With this procedure alcohol concentrations from 10 to 80% can be reached with no damage to the crystals. Since a number of different solvents were available to us, we based our decision on the normality of kinetic behaviour of the enzyme in the solvent and its fluidity at low temperatures. Our choice was methanol–water (70%:30%, v/v), a mixture of very low freezing point with a viscosity very close to pure water, even at -75°C (ref. 5).

Data collection and substrate binding

To obtain stable enzyme–substrate complexes, the substrate must be flowed into the crystal at sub-zero temperature. Richards and his colleagues have described a flow cell for use with single-crystal X-ray diffractometers that holds the crystal firmly in place while allowing liquid to flow through it continuously¹⁴. We have used this device both for substrate binding and solvent transfer experiments. In practice, the crystal is mounted in the flow cell in 0.01 M acetate ($p\text{H} 5.0$). It is then cooled to 5°C on the diffractometer and the liquid is replaced by 20% methanol–80% water at the same protonic activity. The temperature is then lowered 10°C and the liquid is replaced with one of 10% higher methanol concentration. It takes about 7 min for the wave front of fresh liquid to reach the crystal, and equilibration at each temperature/alcohol stage takes 10 to 15 min. For substrate binding at -55°C a flow rate of about 2 ml d^{-1} was used, with the substrate dissolved in 70% methanol and flowed over the crystal in the usual way.

Data were collected on a Syntex P2₁ diffractometer specially modified for protein crystallography (G.A.P. and D.T., unpublished), equipped with a Syntex LT-1 low temperature device which was also extensively modified (G.A.P., unpublished). Data collection was by Wyckoff step scan¹⁴, which enabled us to collect 2,400 reflections per day. All low temperature work was done at $-55 \pm 4^\circ\text{C}$.

The substrate chosen for this study was *N*-carbobenzoxy-L-alanyl-*p*-nitrophenyl ester (ZAP), as this was the substrate about which the most kinetic information was available¹¹. It was dissolved in 70% methanol, 0.01 M acetate ($p\text{H}^* 5.2$) at $3 \times 10^{-3} \text{ M}$. To avoid problems due to its spontaneous hydrolysis the solution was made fresh twice daily and kept at -35°C .

The use of the diffractometer–flow cell system made it possible to follow the binding of ZAP crystallographically. A small set of medium intensity reflections at high resolution were monitored at regular intervals. Plots of their intensity with time were monotonic and could be interpreted in terms of a first-order process with an overall rate constant of $5.4 \pm 1.3 \times 10^{-5} \text{ s}^{-1}$ (Fig. 1). This is in excellent agreement with the acylation rate constant measured for the ZAP–elastase crystal system at -50°C by Ahmed and Fink¹¹, if the value is adjusted for the difference in substrate

Table 1 Refined heavy-atom parameters and phasing statistics

Derivative	Coordinates*			Occupancy†	Temperature factor	R_c ‡	$\langle f_H \rangle$ §	$E^ $
Uranyl nitrate (5 mM, 2 weeks soak at -25°C)	x 0.1170	y 0.5851	z 0.3962	92	A^2 33.1	0.48	88	40
Sodium mersalyl (3 mM, 2 weeks soak at $+25^\circ\text{C}$)	0.0311	0.2666	0.0637	65	25.8	0.53	104	62
	0.0449	0.9713	0.2125	30	10.8			
	0.1763	0.7747	0.0082	61	155.4			

* x , y and z are the heavy-atom coordinates in one asymmetric unit (one elastase molecule) in fractions of the unit cell edges.

†Occupancy in electrons on an approximate absolute scale.

‡ R_c is the Cullis R -factor $\frac{\sum |(F_{PH}-F_P)|-f_H|}{\sum |(F_{PH}-F_P)|}$ the sums being over all centric reflections¹⁹.

§ $\langle f_H \rangle$ is the r.m.s. heavy-atom structure amplitude in electrons.

|| E is the r.m.s. lack of closure of the phase triangle at the most probable phase, in electrons²⁰.

The mean figure-of-merit²¹ for the 2,981 reflections to 3.5 Å is 0.73.

concentration (we observed that the rate varied linearly with substrate concentration, as expected). Collection of a complete set of three-dimensional data to 3.5-Å resolution was begun after the binding curves had levelled off; two days were required to collect the set. Neither low temperature nor substrate binding caused any damage to the elastase crystals; indeed, the mosaic spread of the crystal decreasing by 25% on cooling. The unit cell parameters of the enzyme in alcohol at -55°C and the enzyme-substrate complex at the same conditions agreed within 0.5% with those of the enzyme in water at room temperature ($a=51.5$ Å, $b=58.0$ Å, $c=75.5$ Å, $\alpha=\beta=\gamma=90^\circ$, space group $P2_12_12_1$), suggesting that the three cases were mutually isomorphous.

Phase determination

The crystallographic binding curves suggested that substrate was binding to the crystalline enzyme at low temperature, but to interpret the binding, a set of phase angles for the native enzyme structure was required. Phases were available from the earlier room temperature structure determination¹², but we did not use them. The room temperature structure was of an inhibited enzyme; moreover, one of the heavy-atom derivatives used to solve the structure was itself a covalent inhibitor. The phase angles from this structure determination therefore might be biased towards the presence of material in the substrate binding site. We decided to obtain phase information free from possible

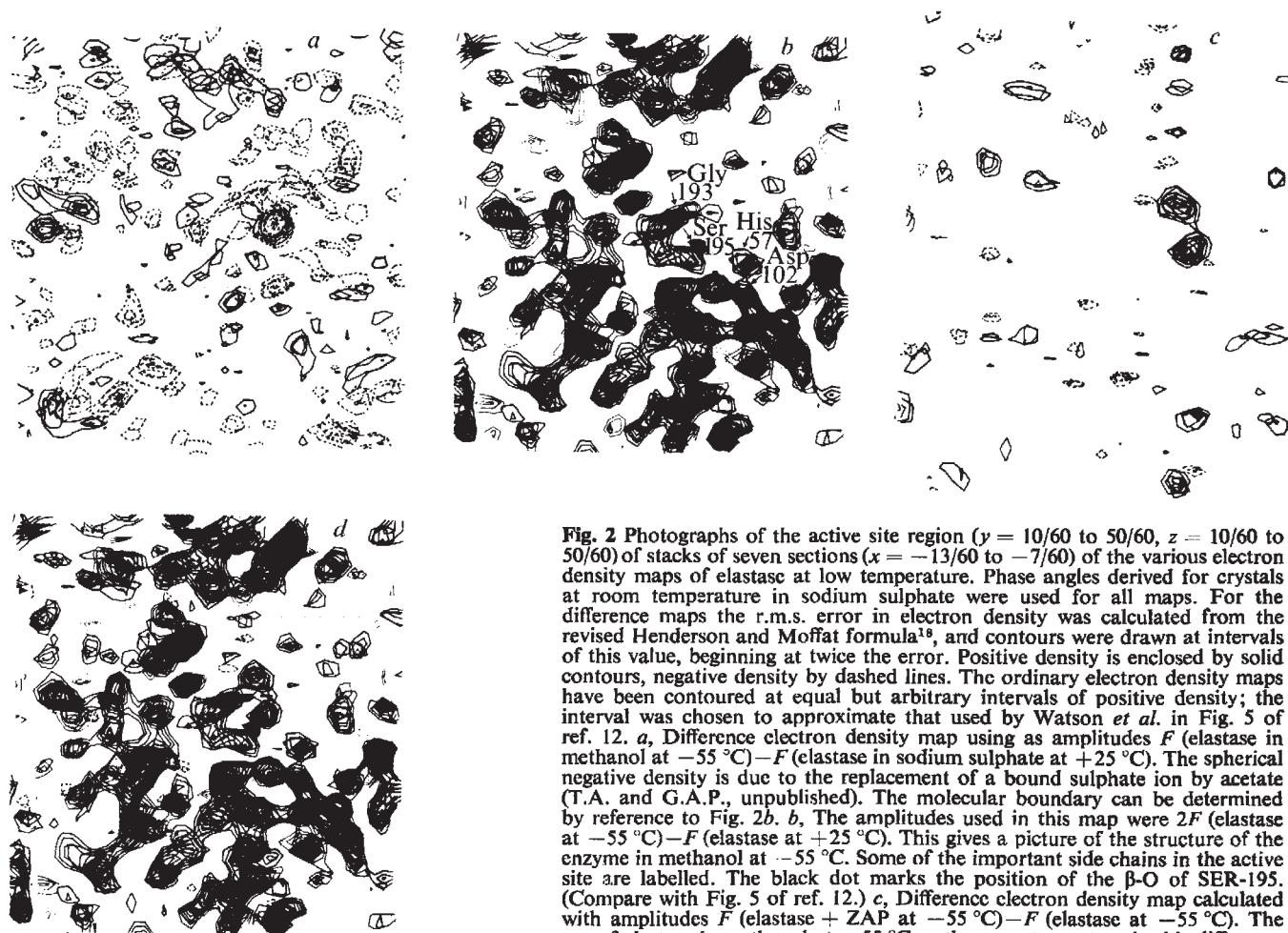


Fig. 2 Photographs of the active site region ($y = 10/60$ to $50/60$, $z = 10/60$ to $50/60$) of stacks of seven sections ($x = -13/60$ to $-7/60$) of the various electron density maps of elastase at low temperature. Phase angles derived for crystals at room temperature in sodium sulphate were used for all maps. For the difference maps the r.m.s. error in electron density was calculated from the revised Henderson and Moffat formula¹⁸, and contours were drawn at intervals of this value, beginning at twice the error. Positive density is enclosed by solid contours, negative density by dashed lines. The ordinary electron density maps have been contoured at equal but arbitrary intervals of positive density; the interval was chosen to approximate that used by Watson *et al.* in Fig. 5 of ref. 12. *a*, Difference electron density map using as amplitudes F (elastase in methanol at -55°C) $- F$ (elastase in sodium sulphate at $+25^\circ\text{C}$). The spherical negative density is due to the replacement of a bound sulphate ion by acetate (T.A. and G.A.P., unpublished). The molecular boundary can be determined by reference to Fig. 2b. *b*, The amplitudes used in this map were $2F$ (elastase at -55°C) $- F$ (elastase at $+25^\circ\text{C}$). This gives a picture of the structure of the enzyme in methanol at -55°C . Some of the important side chains in the active site are labelled. The black dot marks the position of the β -O of SER-195. (Compare with Fig. 5 of ref. 12.) *c*, Difference electron density map calculated with amplitudes F (elastase + ZAP at -55°C) $- F$ (elastase at -55°C). The use of elastase in methanol at -55°C as the parent structure in this difference map cancels out the high noise in the solvent region seen in Fig. 2a. *d*, The difference map of *c* superimposed on *b*, showing the interactions the substrate makes with the enzyme. The methyl group of the alanyl portion of the substrate points into the paper, where it is in contact with the methyl group of VAL-216.

bias by solving the structure of the uninhibited enzyme at 3.5-Å resolution using heavy-atom derivatives which do not bind in the substrate binding site. Two were quickly found, one of which, uranyl nitrate, was used in the earlier study¹². All phase determination was done on the enzyme in 1.2 M sodium sulphate (pH 5.0) at +25 °C for reasons of speed and convenience. Details of the phasing will be published separately. Though not as high in quality as the earlier work, the phase determination was acceptable (Table 1). These phases for the uninhibited native enzyme in sodium sulphate were used for all electron density calculations.

Electron density maps

In addition to the room temperature data collected to phase the structure amplitudes, complete sets of data to 3.5-Å resolution were collected on elastase in 70% methanol at -55 °C and elastase + ZAP in 70% methanol at -55 °C. An electron density map of the native enzyme at +25 °C in sodium sulphate was virtually identical to that obtained by Watson and colleagues on the inhibited enzyme¹². The only significant differences were the absence of the inhibitor density, the presence of a dense spherical feature in the active site interpreted as a bound sulphate ion¹⁵, and the repositioning of HIS-57 to form a complete charge-relay system identical to that observed in chymotrypsin¹⁶ (the histidine is prevented from assuming this position when the inhibitor is bound).

Two electron density maps were calculated for native elastase in methanol at -55 °C. The first, a difference electron density map between the low temperature and room temperature crystals, has as its largest feature a hole at the position of the bound sulphate ion, indicating that this ion is lost in the salt-free mixed solvent, as expected (Fig. 2a). There are no significant features within the molecular envelope, although there are numerous peaks and holes in the solvent-filled interstice. The only significant positive features are on the surface of the molecule, and we have interpreted these in some cases as slight shifts in the position of side chains, in others as the appearance of side-chain density where none was visible at room temperature (presumably because of thermal vibration). The second map shows the electron density of elastase in 70% methanol at -55 °C. The positions of important amino acids in the active site region were noted in this map (Fig. 2b).

We next calculated a difference Fourier synthesis which showed the difference in electron density between elastase and elastase + ZAP, both in 70% methanol at -55 °C. The only significant feature was a bilobed peak in the active site region (Fig. 2c). One of the lobes was within covalent bond distance from the β -oxygen of SER-195; the other, a planar density feature, was interpreted as the benzene ring of the carbobenzoxy group. There was no density for the *p*-nitrophenol portion of the substrate. An integrated electron count of the peak indicated approximately 80% occupancy for the substrate, in excellent agreement with the value found by Fink and Ahmed¹¹. When this difference map was superimposed on the electron density map of the unsubstituted enzyme at -55 °C, the close contact with SER-195 was obvious (Fig. 2d; the black dot in the centre of the photograph is the β -oxygen position as determined by Watson and his colleagues at 2.5-Å resolution¹⁷). We interpret these maps as depicting a stable, productive acyl-enzyme intermediate in the catalytic process, as predicted by Fink and Ahmed in the accompanying article¹¹. We believe that the substrate was bound to the crystalline enzyme, that *p*-nitrophenol was split off when the alanyl residue became covalently attached to the β -oxygen of SER-195, and that deacylation was prevented from occurring by the low temperature used in the study. To test this interpretation, we attempted to wash the crystal free of bound substrate at -55 °C. After 4 d of continuous flow of substrate-free 70% methanol a set of data was collected

and another difference Fourier synthesis was calculated. The results showed no reduction in the amount of bound substrate, suggesting that the mode of attachment was covalent. When the temperature was raised to -10 °C, the intensity binding curves reversed, suggesting loss of bound substrate. Another set of data was collected after 20 h at the elevated temperature, and this showed no detectable substrate remaining in the active site.

Low temperature protein crystallography

We do not believe it is appropriate, at this stage of the analysis with its modest resolution, to use these results to answer detailed questions about the mechanism of serine protease catalysis or the nature of the catalytic power of enzymes. We hope that some insights into these questions will come from the extension of this work to much higher resolution and several different substrates. For example, since the rate-limiting step for the hydrolysis of *p*-nitroanilide substrates is the formation of the acyl enzyme rather than its breakdown, binding a substrate of this type to the crystalline enzyme at low temperature may yield a pre-acyl enzyme intermediate or the Michaelis complex¹¹. This would give a second "stop-action" photograph of a different point in the overall reaction pathway. Our present study, combined with the work of Fink and Ahmed in the accompanying article¹¹, which was essential for the planning and interpretation of our experiments, demonstrates that it is feasible to use low temperature protein crystallography to stabilise and study actual enzyme-substrate complexes. We believe that the technique has great potential to reveal the atomic details of enzyme catalysis, especially when it can be applied to several substrates with different rate-determining steps. In conjunction with kinetic and spectroscopic studies of crystalline and dissolved enzymes^{22,26}, this technique offers the hope of increased understanding of the events in the catalysis of reactions by enzymes. We believe that cryoenzymology can be applied to a wide variety of crystallisable enzymes.

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letters to nature

Do freely falling bodies radiate?

It seems clear that, although gravitational radiation has not yet been detected, its existence is predicted by general relativity. Whether freely falling bodies, that is, bodies moving under gravitation alone, radiate gravitational waves is, however, quite unclear. This is an important question because most astronomical motion is free fall, and if radiation is not produced by this, there will be much less of it present than some investigators believe. The situation has been clearly explained by Ehlers, Rosenblum, Goldberg and Havas (unpublished). Briefly, the doubts arise because those approximation methods which quite convincingly predict gravitational radiation from certain moving systems do not apply to bodies in free fall.

The question could be answered by suitable exact solutions of Einstein's equations. Until recently, however, the known exact solutions for freely falling matter have been spherically, plane or cylindrically symmetric or have been homogeneous: that is, they have had too much symmetry to be realistic models for radiating systems.

An exact solution has now been discovered¹ which is sufficiently unsymmetric to throw important light on the subject. It is a solution of Einstein's equations for dust, which is, of course, matter under no forces except gravity. The metric is

$$ds^2 = -e^{\lambda} dr^2 - e^{\omega} (dy^2 + dz^2) + dt^2 \quad (1)$$

where

$$e^{i\omega} = \frac{\varphi(r,t)}{P(r,y,z)}; e^{i\lambda} = \frac{P}{W(r)} \frac{\partial}{\partial r} e^{i\omega} \quad (2)$$

$$P = a(r)(y^2 + z^2) + 2f(r)y + 2g(r)z + c(r) \quad (3)$$

$$ac - f^2 - g^2 = \frac{1}{4} \quad (4)$$

φ is a Friedmann function, that is, it satisfies the Friedmann equation

$$\dot{\varphi}^2 = [W(r)]^2 - 1 + \varphi^{-1} S(r) \quad (5)$$

where the dot means $\partial/\partial t$; a, f, g, c, W, S are arbitrary subject to equation (4), and a further arbitrary function arises in the integration of equation (5). The density ρ is given by

$$8\pi\rho = \frac{PS' - 3SP'}{\varphi^2(P\varphi' - P'\varphi)} \quad (6)$$

where the prime means $\partial/\partial r$. By a suitable choice of arbitrary functions the density may be made positive everywhere; and it can also be made to fall off with r as rapidly as one likes, so the solution may be thought of as referring to a cloud of dust concentrated towards the centre but extending very tenuously to infinity.

The motion of the dust, governed by equation (5), is one of expansion or collapse, as in the Friedmann cosmological models, but dependent on the radial coordinate r . The two-dimensional surfaces $r = \text{constant}$, $t = \text{constant}$ are spheres and y, z are stereographic coordinates. The spheres of different radii are not concentric, however, and taken as a whole the three-

dimensional space of r, y and z is not spherically symmetrical. In fact the solution has no Killing vectors whatever for general values of the arbitrary functions (W. B. B., A. H. Sulaiman and N. Tomimura, unpublished).

Does the space-time contain gravitational radiation? It is not easy to answer this question: there is no conclusive mathematical test for the presence of radiation in a space-time, and to examine the solution for a radiative flux at spatial infinity turns out to be difficult because of the comoving coordinate system in which the solution is formulated. The following argument seems conclusive, however. Consider the portion of the solution (1)–(6) contained within $0 \leq r < r_0$ ($r_0 = \text{constant}$). I have shown (unpublished) that it is possible to match this portion to a Schwarzschild metric which is valid for $r > r_0$. The Schwarzschild metric being static there is no radiation passing through it, so the moving dust in the region $0 \leq r < r_0$ is not radiating.

It would be quite wrong to argue from this that freely falling matter never radiates. The investigation does, however, show the existence of a class of non-radiative motions of dust in a space-time without Killing vector symmetries.

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Globular clusters as a source of X-ray emission from the neighbourhood of M87

At least five galactic X-ray sources have been identified with globular clusters^{1–3}, and it is possible that a similar association holds for the bright unidentified sources near the galactic centre⁴. Intense X-ray bursts have been detected⁵ from the direction of one of these (3U1820–30/NGC 6624), and a star cluster, probably globular, has been found⁶ in the error box^{7,8} of the so-called 'rapid burster', MXB1730–335. Moreover, the error box of a hard X-ray flare observed from the Cosmos 428 satellite⁹ contains NGC5904 (M5). This globular cluster has similar properties¹⁰ to the others that emit X rays, and if normally weak it is unlikely to have appeared in the Uhuru catalogue because of its position. The X-ray emission from globular clusters may be attributable to accretion on to compact objects, the accreting material being supplied from binary companions^{11–14}, or gas trapped in the potential well of the cluster^{15,16}.

There are relatively few intrinsically bright optically observed globular clusters in our Galaxy. Counts of objects in the vicinity of M87, however, reveal that it has an extensive halo of globular clusters^{17,18}, the number of which may exceed 10,000 within a radius of 23 arc min (ref. 19). The greater number of bright globular clusters in M87 may be explicable as a population effect¹⁸. The similarities

between the optical properties of these globular clusters and those in our own Galaxy suggest that the former may also contain X-ray sources.

The brighter globular clusters in M87 may also be substantially more X-ray luminous. The presence of a massive black hole or of captured binaries depends sensitively on the properties and past history of the core of the globular cluster. Gas to fuel a massive black hole, or single neutron stars, in a globular cluster orbiting our Galaxy will be stripped on passing through the galactic disk, and possibly even through the halo²⁰. This stripping will not occur in an elliptical galaxy; consequently, there may be proportionally more gas available in identical globular clusters in M87 than in our Galaxy.

On either hypothesis, the average X-ray luminosity of individual globular clusters may be of the order of 10^{38} erg s⁻¹. This raises the possibility that the integrated globular cluster emission may account for a substantial fraction of the X-ray emission observed from the region of M87 ($L_x \sim 7 \times 10^{43}$ erg s⁻¹; ref. 21). (Katz²² has suggested that the X-ray emission from clusters of galaxies may be attributable to unspecified compact sources.) In support of this proposition we note that the extended X-ray emission^{21,23} from the Virgo cluster is centred on M87, which lies ~ 45 arc min from the cluster centroid. We expect that the general X-ray emission from the globular cluster will appear to be smoothly and symmetrically distributed about M87 at moderate spatial resolution. A few clusters of higher luminosity might be resolvable with currently proposed imaging X-ray telescopes.

A similar situation may apply to the elliptical galaxy NGC3311 in Abell 1060, which as a cluster has been suggested as the identification for the X-ray source 3U1044-30^{1,24}. It seems possible that that galaxy is surrounded by a similar globular cluster population to that of M87 (ref. 25).

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Pre-terrestrial shear faulting and heat treatment of the Jamestown iron meteorite

We have made a metallographic examination of a 250-g slice of the Jamestown iron meteorite. The meteorite was first reported by Huntington¹ as a mass of 4 kg, with a dishd,

flake-like shape of maximum dimensions $26 \times 13 \times 3.7$ cm, and with two entirely different external surfaces—a smooth convex side, and a slaggy, vesicular, concave side, pitted by circular cavities up to 2 cm in diameter. Buchwald² has reported on the general metallography of the Jamestown Iron but does not record which of the many sections of this meteorite his description is based on.

Our British Museum specimen (BM 67215) mates with that shown in Fig. 3 of ref. 1. Most of our general microscopic observations are in agreement with those of Buchwald². We found that phosphide is absent, daubreelite is present as rare particles, and troilite is present in varying degrees of shock alteration. The size and shape of the ϵ -kamacite, taenite and plessite components are consistent with membership of chemical group IVA in the classification scheme of iron meteorites.

Furthermore, the convex surface of the British Museum specimen retains relics of a heat alteration zone, produced by ablative burning during atmospheric entry. The concave surface shows somewhat less penetration of heat alteration but instead shows a considerable accumulation of the products of ablation melting, which Buchwald² describes as fine grained metallic eutectics. The Jamestown meteorite must have traversed the Earth's atmosphere as an oriented projectile with the convex surface leading.

The surface heat alteration overlies all other aspects of metallic macro- and microstructure which must therefore be of pre-terrestrial origin and not produced by impact with the surface of the Earth. Buchwald² has noted that the ϵ -kamacite must have been reheated. Though it is not easy to specify the exact conditions of heat treatment, some limits may be proposed, since on low power examination the kamacite seems to consist of the acicular ϵ structure noted by Jain and Lipschutz³, but our examination at higher magnification shows that the ϵ within the bulk of the kamacite is diffused but not recrystallised. This absence of recrystallisation in the bulk of the kamacite suggests a reheating temperature below 450 °C, since our laboratory heat treatment of the shock-hardened kamacite of Trenton does show detectable recrystallisation at this temperature.

Buchwald² noted local distortion, shearing and folding of plessite and taenite, but did not indicate the scale of these effects and did not examine the detailed metallography of the shearing, which in some instances is encountered on a macroscopic, not a local, scale. Indeed, the outstanding feature of the etched macrostructure of the British Museum specimen is the interruption to the regularity of the Widmanstätten pattern by the traces of at least five shear-displacement surfaces, of which the longest extends for 6 cm and leaves the meteorite at two points on the leading convex edge. Others extend from the trailing concave surface inwards for about 2 cm. One of the latter has developed into the shear-displacement type of crack that Axon and Steele-Perkins⁴ have reported in fragments of the Henbury Iron. It is, however, important to recognise that the shear-displacements in the Henbury specimen were produced by the crater-forming impact of the meteorite with the Earth's surface; but the shear-displacement crack in the Jamestown Iron is invaded by ablation melt product and is therefore of pre-terrestrial origin.

Attention has been directed to the absence of recrystallisation in the bulk of the ϵ -kamacite, and a reheating temperature < 450 °C deduced. By contrast, the more heavily deformed material within and immediately adjacent to the surfaces of shear displacement in the Jamestown Iron has recrystallised. The zones of finely recrystallised kamacite extend on both sides of the shear surface to a width of about 0.15 mm, and it is this band of recrystallisation that accentuates the appearance of shear surfaces in the macrostructure. This local recrystallisation of the heavily sheared material is consistent with the proposed reheating temperature, and explains why the macroscopically visible thickness of the shear traces (~ 0.15 mm) in the Jamestown Iron is greater than in other meteorites previously examined by us.

The metallographic features of the Jamestown meteorite would be consistent with the following history. After a period of slow cooling (approximately $300^{\circ}\text{C Myr}^{-1}$, according to the bandwidths reported by Buchwald²) in which the Widmanstätten structure developed, the material formed part of a massive projectile that impacted, and formed a crater on, some unknown planetesimal in the Solar System. The present Jamestown meteorite is part of a fragment that was produced during that crater-forming event. It shows shear displacement surfaces and the development of fracture by separation along the surfaces of shear faults in a manner similar to that noted by Axon and Steel-Perkins⁴ for the terrestrial impact of the Henbury Iron. After the crater-forming impact the Jamestown material was heated to a temperature below 450°C , probably by a blanket of hot ejecta on the surface of the unknown planetesimal. At some later stage it was released from its intermediate host, circulated in space and eventually entered the Earth's atmosphere, where it acquired its heat alteration zone and an accumulation of ablation deposit, some of which penetrated a pre-existing shear fissure on the rear surface of the oriented projectile.

Shock effects are common in group IVA irons, and Jain and Lipschutz³ have noted the presence of reheating effects after shock in the Boogaldi, Western Arkansas, Huizopa, Charlotte, Yanhuaitan, Maria Elena and Social Circle meteorites. The massive shear traces in the Jamestown Iron are, however, so far unique within the group IVA irons, although the Seneca Township meteorite shows a similarly diffused ϵ structure in its bulk.

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Lead and radium in the lower stratosphere

MORE than a decade ago Junge¹ found that the concentration of particles in the stratosphere is greater than in the upper troposphere, and that their main chemical constituents were ammonium and sodium sulphates, thought to arise from the ascent of their volatile precursors H_2S and SO_2 (ref. 2). In addition, minute concentrations of aluminium, calcium, chlorine, chromium, cobalt, copper, gold, iron, manganese, silicon and titanium have been detected^{3,4}, which, having no volatile precursors, enter the stratosphere as particles. As the extra-terrestrial fraction in stratospheric aerosols is extremely small⁵, their main source seems to be the surface of the Earth, but data available on the nature of such sources, and on the chemical composition and concentration of stratospheric aerosols are rather scarce—an astonishing fact, in view of their possible climatic impact. With this in mind, we have measured the concentrations of stable lead, ^{226}Ra and its radioactive daughter ^{210}Pb at various heights. These substances enter the atmosphere from several surface sources, both natural and artificial⁶. The concentration of ^{226}Ra in particulate materials such as soil dust or fly ash is well known, and may be used for the assessment of the stratospheric content of this kind of dust.

During the past three years we collected 112 samples of

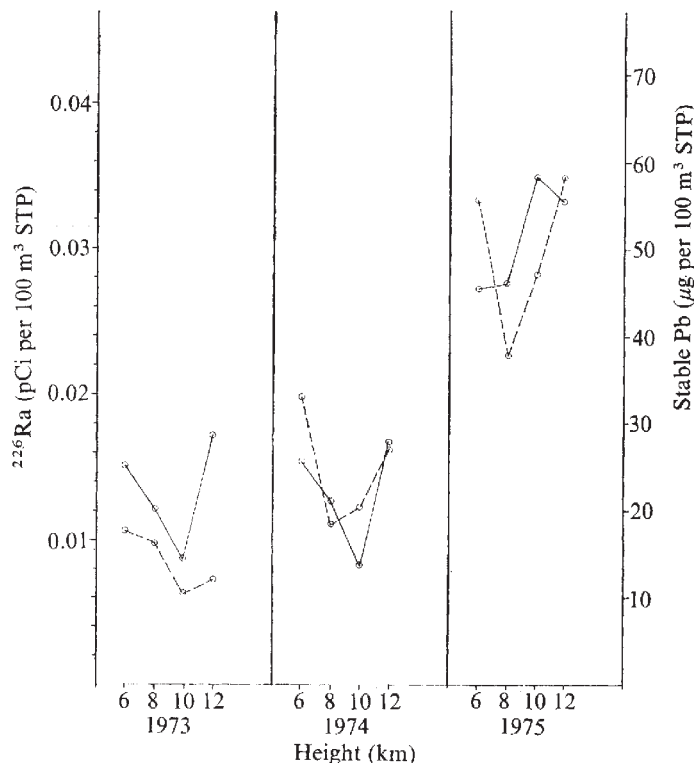


Fig. 1 Vertical distribution of ^{226}Ra (---) and stable Pb (—) in the atmosphere.

aerosols over Poland, from heights of 6, 8, 10 and 12 km. At each altitude 9–15 samples were collected in 1973, 6–9 in 1974 and 6–11 samples in 1975. The levels of the tropopause for each sampling profile were determined from data on the vertical gradient of temperature, supplied by the Institute of Hydrometeorology, Warsaw.

We used 1,600-cm² PVC fibre filters, type FPP-15-1.7 (Soviet made), placed in collectors suspended under the wings of LIM-type jet planes. After wet-ashing and dissolving in the filters, the quantities of ^{226}Ra , stable Pb, ^{210}Pb , ^{90}Sr and ^{137}Cs collected were determined, using methods described elsewhere⁷.

In Fig. 1 we present the annual mean air concentrations of stable Pb and ^{226}Ra , found between July and November 1973, May and October 1974 and March and December 1975. The striking feature is the high concentration (comparable with ground level) of ^{226}Ra and stable Pb at 12 km. In 1973 the mean ^{226}Ra concentration at 12 km was 0.7×10^{-4} pCi m⁻³ STP, 1.6×10^{-4} pCi m⁻³ STP in 1974 and 3.5×10^{-4} pCi m⁻³ STP in 1975. The mean stratospheric stable-Pb concentrations were 29×10^{-2} μg m⁻³ STP in 1973 and 1974 and 55.7×10^{-2} μg m⁻³ STP in 1975. The concentrations of ^{226}Ra which we found in Warsaw in the air at ground level range from 1.5×10^{-4} to 6.0×10^{-4} pCi m⁻³ and with the mean stable-Pb concentration of 10.0×10^{-2} μg m⁻³ in non-urban air⁸.

Comparing Figs 1 and 2, it is also interesting to note that the vertical gradients of ^{226}Ra and stable-Pb concentrations in the troposphere are opposite to the gradients of the fission products. The concentrations of ^{226}Ra and Pb decrease with altitude between 6 and 10 km, reaching minima below the tropopause, and increasing sharply above it. This shows the ground level origin of these substances, and indicates the existence of trapping in the stratosphere.

Stable Pb, ^{226}Ra and ^{210}Pb have been discharged into the atmosphere by natural sources since time immemorial. Volcanic activity, forest fires and the action of the wind on soil and sea water are estimated to contribute $\sim 5.4 \times 10^{-4}$ μg of Pb m⁻³ of atmospheric air⁹ and 3×10^{-9} pCi m⁻³ of ^{226}Ra (ref. 6). But the present concentrations of stable Pb and ^{226}Ra in the stratosphere are 2–3 and 5 orders of magnitude higher than these estimated natural levels. It follows that the bulk of

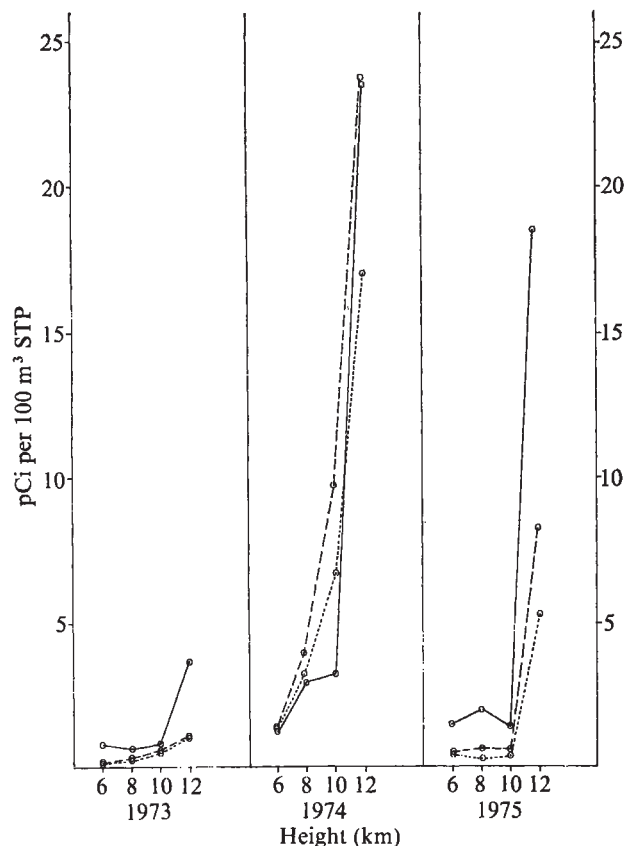


Fig. 2 Vertical distribution of ^{210}Pb (—), ^{137}Cs (---) and ^{90}Sr (····) in the atmosphere.

these nuclides in the stratosphere is probably of artificial origin, unless the estimation of their natural input is wrong. Coal burning, phosphate fertilisers and cement production are the main man-made sources of ^{226}Ra in the atmosphere; their global contributions have been calculated as 150, 400 and 1.8 Ci yr^{-1} respectively⁶.

If the mean concentration of ^{226}Ra in the $\sim 10^{10} \text{ m}^3$ of atmosphere up to 20 km, were similar to that found in the lower stratosphere, that is $\sim 1.0 \times 10^{-4} \text{ pCi m}^{-3}$, then the total content of ^{226}Ra in this volume would be $\sim 1,000 \text{ Ci}$, a value not much different than the annual contribution from artificial sources.

The main artificial sources of stable Pb are the combustion of lead alkyls in petrol, which contributes $120,000 \text{ t yr}^{-1}$ (Northern Hemisphere)¹⁰ and the burning of coal, contributing $3,000 \text{ t yr}^{-1}$ (whole world)⁸. For the same volume of atmosphere, with a mean Pb concentration of $1.0 \times 10^{-2} \mu\text{g m}^{-3}$, the total lead content would be 100,000 t. These tentative calculations support the suggestion that most of ^{226}Ra and Pb in the stratosphere is of artificial origin.

During the years 1973 and 1974 a series of Chinese and French nuclear explosions has been conducted in the atmosphere, which is responsible for the increase of ^{137}Cs and ^{90}Sr in our samples in 1974 and 1975. It is interesting to see in Fig. 2 that ^{210}Pb concentrations in 1974 increased in parallel with those of fission products. In the first half of 1975, ^{210}Pb concentrations increased above the 1974 level, whereas a substantial decrease of ^{137}Cs and ^{90}Sr was observed. At the same time we found a sharp increase in the stable Pb content in both troposphere and stratosphere, seeming to suggest a common source for both types of lead. This is, however, improbable, as the specific activity of lead collected by us in the lower stratosphere is extremely high, reaching, in 1975, $\sim 330,000 \text{ pCi }^{210}\text{Pb}$ per g stable lead, and, in 1974, $\sim 2,400,000 \text{ pCi g}^{-1}$, 3–6 orders of magnitude above the specific activities of contemporary common lead, which range from 1.5 to 501 pCi g^{-1}

(ref. 12). This indicates that common Pb in the atmosphere is of no importance as a source of ^{210}Pb .

From the known concentrations of ^{226}Ra and stable Pb in coal^{6,13}, one can infer that the specific activity of lead in coal is $\sim 30,000 \text{ pCi per g lead}$. In soil this specific activity is $15,000 \text{ pCi per g lead}^{14}$. It follows that although coal and soil might contribute to stratospheric load of ^{210}Pb , the bulk of it comes from other sources. Coal burning contributes yearly $\sim 200 \text{ Ci}$, and phosphate fertilisers 150 Ci (ref. 6). Natural production of ^{210}Pb in the atmosphere from ^{222}Rn , estimated as $\sim 0.62 \text{ MCi yr}^{-1}$ (ref. 12), clearly overwhelms these sources. Nuclear explosions are suggested to have produced fractions of a MCi of ^{210}Pb during the 1958–59 and 1961–62 test series, by the reaction $^{208}\text{Pb}(2n, \gamma)^{210}\text{Pb}$, from the lead content of the construction of bombs^{14,15}. This contribution, being transient and of short duration, would explain the excess peak concentrations of ^{210}Pb in the stratosphere accompanied by high concentrations of fission products as observed by us and others (P. W. Krey, unpublished). It also explains similar correlations found in fossil ice collected at glaciers in the Tatra Mts, Poland, the Jotunheimen Mts, Norway and the Himalayas, Nepal¹⁴.

The minute industrial sources of ^{210}Pb , cited above, may locally influence the content of this nuclide in air and precipitation, as suggested by several authors^{6,14,16}. But the contributions from nuclear explosions, could affect the ^{210}Pb concentration on a global scale. This restricts the use of ^{210}Pb as a tracer in determinations of the age of glacier ice and in atmospheric transport studies.

If the ^{226}Ra content in stratospheric particulates were the same as in the fly ash or soil, $\sim 1\text{--}5 \text{ pCi g}^{-1}$ (ref. 11), then the 1975 concentration of mineral matter bound to ^{226}Ra would reach $50\text{--}250 \mu\text{g m}^{-3} \text{ STP}$ in stratospheric air, at a height of 12 km. This is a rather high concentration. Were these particulates of artificial origin, it would imply that their accumulation in the stratosphere is a fairly new process, which if continued indefinitely might have an important climatic impact in the future.

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Progressive faunal migration across the Iapetus Ocean

DURING the Lower Palaeozoic, there was a gradual increase in the similarity of the faunas between North America to the west of the Appalachians, western Newfoundland, north-western Ireland and Scotland on the one hand, and coastal

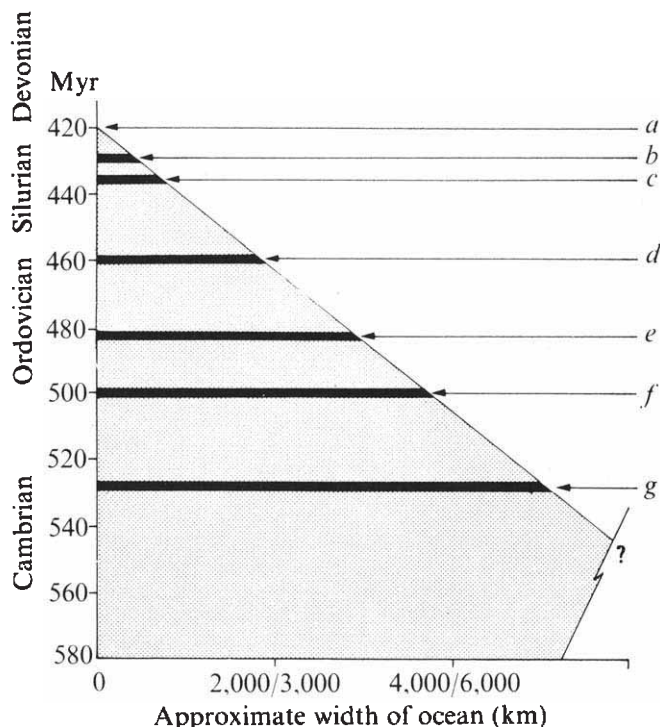


Fig. 1 The times at which faunas became common to both sides of the Lower Palaeozoic Iapetus Ocean. *a*, Closure of ocean (Norway); *b*, freshwater fish; *c*, benthic ostracods; *d*, trilobite and brachiopod species; *e*, trilobite and brachiopod genera (? island hopping); *f*, *Didymograptus bifidus*; *g*, *Dictyonema*.

New England, southern New Brunswick, Nova Scotia, eastern Newfoundland, England and northern Europe on the other hand. The best explanation for this is that the Iapetus (or Proto-Atlantic) Ocean was wide enough to separate two faunal provinces in Cambrian times, and that there was progressive migration of the more mobile components of the faunas as the old ocean closed (Fig. 1). The pelagic animals crossed first, followed later by animals (trilobites and brachiopods) with pelagic larval stages; but animals without a pelagic larval stage (such as benthic ostracods) were not able to cross until the ocean had closed at one point, though not necessarily everywhere along its length. Finally, faunas limited to fresh water or brackish water (like many Devonian fish) did not cross until there were non-marine connections between the continents on either side of the closing ocean.

Pelagic graptolites were among the earliest animals to appear on both sides of the ocean. The many-branched *Dictyonema flabelliforme* migrated across in the Tremadoc; but it was not until the Llanvirn (towards the end of the early Ordovician) that most other graptolites became similar on both sides. The two-branched *Didymograptus bifidus*, for example, appears in the Arenig of Texas, but did not reach England and Wales until the Llanvirn¹.

The next groups of animals to cross were those with pelagic larval stages, notably brachiopods and trilobites. Most genera in these groups were confined to one or other side of the Iapetus Ocean during the Cambrian and early Ordovician; the few cases where genera characteristic of different faunal provinces occurred together (including New World Island, Newfoundland) have been interpreted² as sites of oceanic islands colonised by sporadic spatfalls, which became centres of evolution and migration. The late Ordovician (Caradoc) brachiopod and trilobite faunas show less marked distinctions^{3,4}; though most species are distinct, many genera are common to both sides of the ocean. It is not until the latest Ordovician (latest Ashgill; post-Richmond) that most species of brachiopods and trilobites became identical in Europe and America.

The specific distinctions in the Caradoc and early Ashgill indicate that, at its narrowest point, the Iapetus Ocean must have still been wide enough to prevent free crossing by most trilobite and brachiopod larvae. There are two quite distinct ways of measuring the width of the ocean at this time: by analogy with modern pelagic larva, and by analogy with modern rates of subduction.

Most modern brachiopod larvae have a pelagic stage of less than one week, though it may be as long as three weeks⁵, and 80% of decapod larvae have ranges of between 2 and 7 weeks⁶. But benthic marine invertebrates commonly have a much longer larval life in the tropics, (for example, 7 to 43 weeks for tropical gastropods⁷). Britain and eastern Canada were at about 20°–35°S during the late Ordovician⁸, so we conclude that the late Ordovician brachiopod and trilobite larvae (which seem to have crossed the Iapetus Ocean more or less simultaneously) may have had a larval duration of about 7 to 14 weeks. Data from Thorson⁹ and Scheltema⁷ suggest that larvae with this lifespan could possibly travel across an ocean between 2,000 and 4,000 km wide.

We have estimated that subduction occurred to the north of the Iapetus Ocean (in Scotland and north-western Ireland) between 530 (the age of the Oughterard granite of County Galway) and 390 Myr ago (the Glencoe Caldera of Scotland), a duration of 140 Myr; southward subduction occurred between 490 Myr ago (the Borrowdale Volcanics of northern England) and 440 Myr ago (the Mendip Hills and the Skomer Volcanics). If the average subduction rate during these periods was 4 cm yr⁻¹, then some 7,600 km of ocean crust may have been subducted between the late Cambrian and mid Silurian. Of this, some 4,800 km would have been subducted before the late Ashgill; thus, some 2,800 km of ocean crust might have existed in the late Ashgill. This argument assumes that an active spreading centre no longer existed in the late Ashgillian Iapetus Ocean; if it did exist the ocean could have been narrower. The estimates from pelagic larval migration and from subduction rates thus both suggest that the ocean had a minimum width of around 2,000–3,000 km during the late Ashgill.

The Iapetus Ocean probably did not have parallel margins; there is some evidence to suggest that it may have been wider in eastern Canada and the northern Appalachians than in Europe. The first closure occurred in the north-east⁹, when Greenland and the Baltic Shield collided during the late Silurian. This date is supported by evidence from freshwater fish: the first recorded species to cross the Iapetus Ocean did so between Scotland and Norway during Wenlock or Ludlow times¹⁰.

Subduction of ocean floor continued through the Silurian and early Devonian in the region between Britain and the northern Appalachians, and continental collision in this region (as opposed to Greenland and Norway) did not take place until after a large proportion of the Lower Devonian rocks had formed. The time of collision in this area is clearly related to the Acadian Orogeny, which can be dated as middle Emsian, that is, towards the end of early Devonian time¹¹.

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Chronological evolution of the Kerguelen Islands syenite–granite ring complex

ALKALINE syenites in the Rallier-du-Baty peninsula of the Kerguelen Islands^{1,2} intrude and metamorphose the basaltic lava flows which constitute the major part of the islands³. Recent work⁴ has revealed the existence, in the peninsula, of five ring complexes consisting mainly of syenites containing from 2 to 15% normative quartz (Figs 1 and 2). The southern complex is the largest, and consists of nordmarkites (ring dykes *a, b, c*) and of quartz-rich syenites and alkaline granite (ring dykes *d, e, f*). Some minor intrusive bodies of gabbros (G) and nordmarkites (Σ) are located on the margins of the southern centre (Fig. 2). Chilled margins and enclaves in each individual ring dyke clearly indicate that the sequence of intrusions is centripetal. Furthermore, the contact relationships between the centres show that the magmatic activity has migrated northwards. Previous K–Ar data⁵ have indicated that a syenite from the southern ring complex was emplaced 8.7 ± 0.9 Myr ago, whereas basaltic flows were erupted during the late Oligocene. An additional K–Ar age of 11.5 ± 0.2 Myr was obtained for a

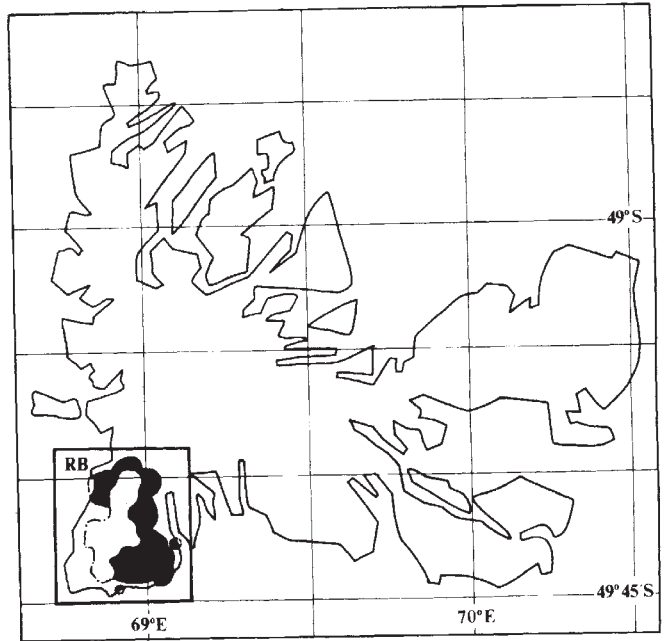


Fig. 1 Location map of the Rallier-du-Baty peninsula (RB) in the Kerguelen Islands.

metabasalt collected near the contact with the syenites and was interpreted⁵ as the minimum value for the time of eruption of the basalts.

Using both K–Ar and Rb–Sr geochronological techniques, we have dated a series of samples, carefully selected from

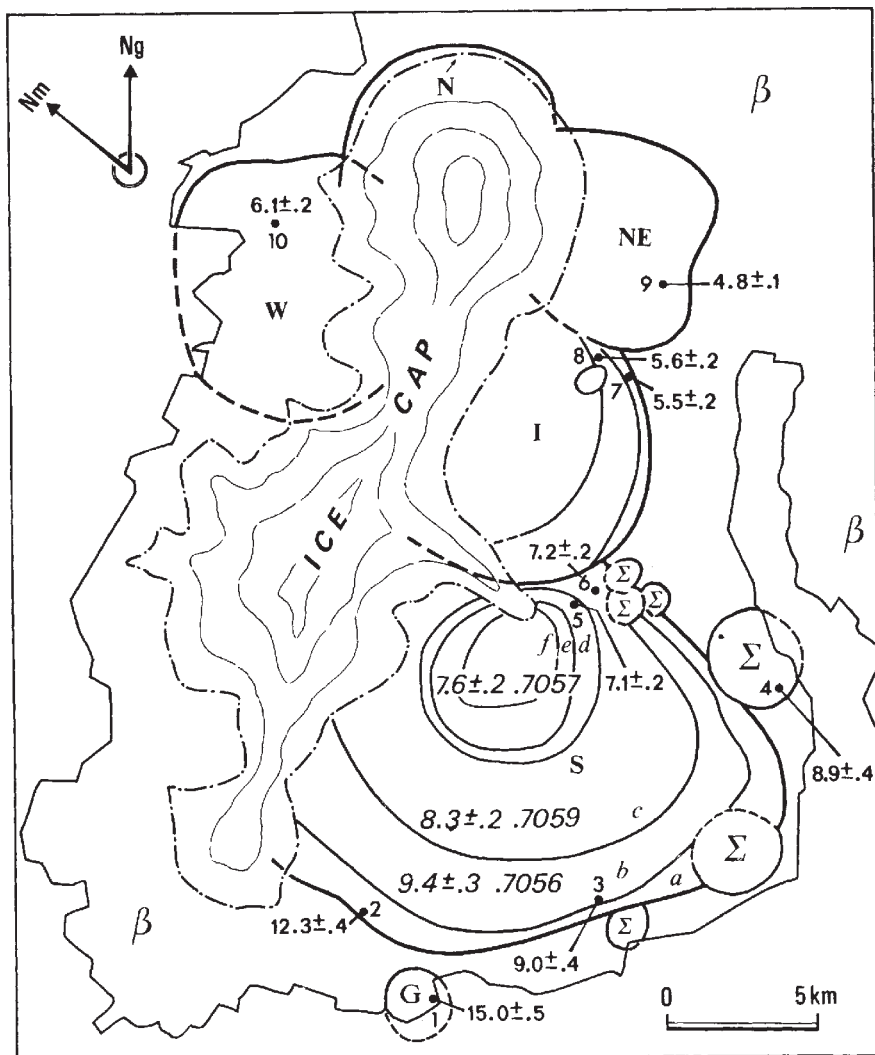
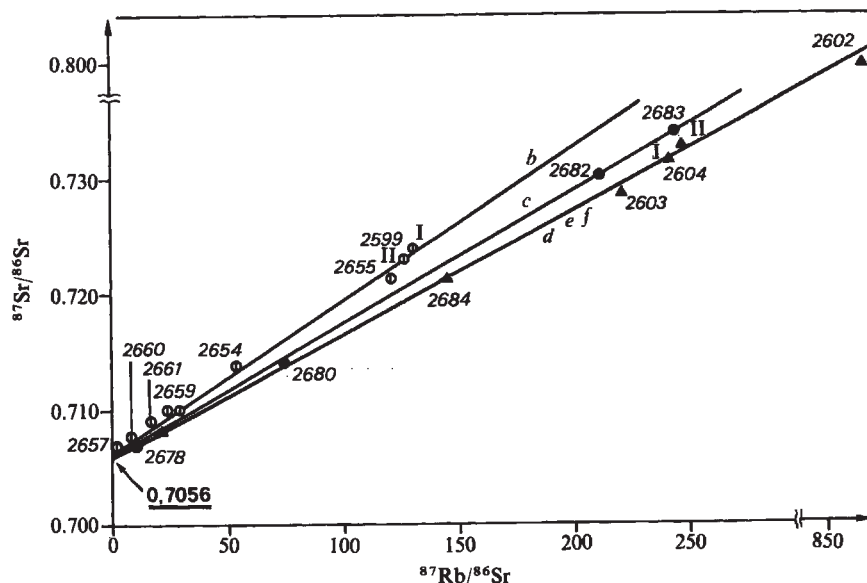


Fig. 2 Geological sketch map of the Rallier-du-Baty peninsula, K–Ar (J.M.C.) and Rb–Sr (L.D., Ph.V.) results. S, Southern centre; I, middle centre; NE, north-eastern centre; N, northern centre; W, western centre. K–Ar results: 1, olivine gabbro; 2, quartz–syenite; 3, aplite dyke; 4, fayalite syenite; 5, granite (chilled margin); 6, microgabbro; 7, quartz–syenite (chilled margin); 8, microgabbro (dyke); 9, syenite; 10, biotite syenite. Rb–Sr results (see Fig. 3). Nm, Magnetic North; Ng, geographical North.

Fig. 3 b, Whole-rock isochron for syenitic ring dyke b (○), 9.4 ± 0.3 Myr; c, whole-rock isochron for syenitic ring dyke c (●) 8.3 ± 0.2 Myr; d, e, f, whole-rock isochron for granitic and syenitic inner ring dykes (▲), 7.6 ± 0.2 Myr. I and II, Duplicate analyses on certain samples.



individual ring dykes in the southern centre (see Fig. 2 for sample localities) and have obtained the following results: (1) Both K-Ar and Rb-Sr methods yield consistent ages ranging from ~ 15 Myr for the minor olivine-gabbro intrusion, and 12 Myr for the outer syenitic ring dyke, to ~ 7 Myr for the inner ones and the core, with an interval of ~ 1 Myr between the emplacement of adjacent ring dykes. (2) Most of the dated samples (Fig. 3) have very high Rb/Sr ratios (up to 300). Three whole-rock Rb-Sr isochrons yielded significantly different ages (9.4 ± 0.3 Myr; 8.3 ± 0.2 Myr; 7.6 ± 0.2 Myr) but they all have very similar initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The 7.6 ± 0.2 Myr isochron, however, combines various samples from the three inner distinct ring dykes (d, e, f) made up of alkaline granites and quartz syenites.

Some additional samples from the western, north-eastern, and intermediate centres have also been measured using the K-Ar method. The ages of the group of complexes range from > 12 Myr in the south, to < 5 Myr in the north-east. These data are in agreement with the deductions made from the field observations and suggest that the magmatic activity migrated at a very slow rate during the Upper Miocene.

The very high, yet variable, Rb/Sr ratios, as well as the radiogenic nature of the samples from the southern centre, allow a fairly precise whole-rock Rb-Sr isochron to be obtained for these very young rocks for the first time. The centripetal age distribution in the southern centre may reflect the consequence of a progressive cooling from the outer ring dyke to the core; however, the presence of chilled margins in the outer part of the ring dykes a, b, c and d (Fig. 2) practically rules out such a possibility. The whole-rock Rb-Sr ages are therefore interpreted as the individual times of crystallisation. The duration of about 5 Myr for the igneous activities in this ring complex is especially informative in terms of the formation of one complete ring complex. Although other similar ring complexes have already been studied in New Hampshire, Nigeria, Niger and Rhodesia⁶⁻⁹, the actual time span for the development of igneous activity in a single ring complex could not be obtained because of the overlap of the analytical errors for the individual ages in successive intrusions.

The initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for the three isochrons are: 0.7059 ± 0.0003 for ring dyke b, 0.7056 ± 0.0004 for ring dyke c, and 0.7057 ± 0.0004 for all three inner ring dykes d, e and f and these values are in agreement with previous data^{10,11}. Using Hedge's measurements, the calculated initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is 0.7055 for three syenitic samples from the southern complex and one sample from a gabbroic intrusion of the Courbet peninsula. We suggest therefore, that the source regions for different ring dykes have the same Sr isotopic composition. Furthermore, the interval between the production of melt and the final crystallisation may have been very short (< 1 Myr in

this case), because, in our opinion, the very high Rb/Sr ratio could produce a very rapid increment in the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in the melt if fractional crystallisation was prolonged. The distinctly high initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios reported⁷ in some geochemically similar rocks (Rb/Sr up to 1,200) from Nigeria which have previously been interpreted in terms of crustal contamination, may have an alternative interpretation if the liquids took a longer time to crystallise finally.

The question of whether continental crust or oceanic crust^{10,11} exists beneath the Kerguelen Islands cannot be resolved from the Sr isotopic data alone. But neither do we have any geochemical or geological (xenolithic) evidence for the presence of continental material under the basalts.

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Native copper in DSDP sediment cores from the Angola Basin

LEG 40 of the Deep Sea Drilling Project (DSDP) drilled two holes in the Angola Basin: site 364 and site 365 (Fig. 1). Native copper was found in two samples (364-5-2, 19–21 cm and 364-5-2, 22–24 cm) from one of the cores taken at site 364. The botryoidal form of most of the copper grains initially suggested formation *in situ* and the subsequent discovery of copper casts of planktonic foraminifera removed any lingering thoughts of contamination. This is not the first report of native copper in DSDP cores. Most other occurrences have, however, been found in basement rocks (for example, site 282) or in the sediments immediately overlying basement rocks (site 105), whence the copper was presumably derived. The copper reported here occurs at least 3 km above volcanic basement rocks, and no copper has yet been found in samples above or below it. Only at site 149 (Venezuelan Basin) has copper previously been found in non-basement-associated sediments. There it occurs as "a number of very small metallic incrustations" in one core of pelagic clay¹.

Site 364 was drilled in a water depth of 2,449 m (see Fig. 1). The copper rests in moderate yellowish brown (10YR 5/4) to light olive grey (5Y 5/3) Miocene marly nannofossil ooze at a depth of ~152 m below the sea floor. Native copper was found in only the samples mentioned, although all other samples at the writer's disposal from this site (364) and the adjacent site (365) were carefully re-examined after copper was found. The sample at 22–24 cm contained five grains of copper whereas the sample at 19–21 cm contained several dozen. My data refer only to the latter sample.

Native copper makes up 12% (by weight) of the total sample. The copper grains are metallic reddish-brown, mostly botryoidal masses, and individually range up to 4 mm across. A number of copper casts of planktonic foraminifera (Fig. 2) are also present (the frontispiece of vol XL, *Initial Reports of the Deep Sea Drilling Project* carries colour photographs of several of these grains). Many of the grains of copper are partially coated with very light green calcium carbonate and clay; most, but not all, of this coating is soluble in dilute HCl. The greenish tint is probably caused by reaction of the copper with the surrounding carbonate-rich sediments, forming copper carbonate.

Table 1 shows that, with the exception of small amounts of sulphur, the grains can be considered wholly copper (the concentrations of the other elements, if real, are negligible). The presence of 0.18% and 0.64% sulphur suggests a very small amount of copper sulphide may be present. The copper value in the < 89- μ m diameter size fraction is obviously vastly higher than in normal marine sediments¹, indicating that minute copper particles are also present in this fine fraction. Concentrations of the other elements in this fraction are more or less normal for marine sediments.

The presence of native copper in any natural environment (whether igneous, metamorphic, or sedimentary) is always somewhat surprising in view of the ease with which copper combines with other elements (especially sulphur). Nevertheless, copper abundantly exists in this form in a diversity of environments, and the literature on the subject is voluminous. Its presence in sedimentary rocks, the concern of this study, is less common than in igneous rocks, but the literature is still extensive^{2,3}.

Onshore erosion and mechanical transport of these particles to site 364 may be discarded as a possible mechanism of origin. It is difficult to imagine transport which would bring only copper grains to this site, without other terrigenous grains as well. Moreover, the copper grains are not rounded and abraded, as would be caused by any transporting mechanism capable of carrying these large grains. Finally, the presence of copper-infilled planktonic foraminifera conclusively proves an *in situ* deposition from copper solutions.

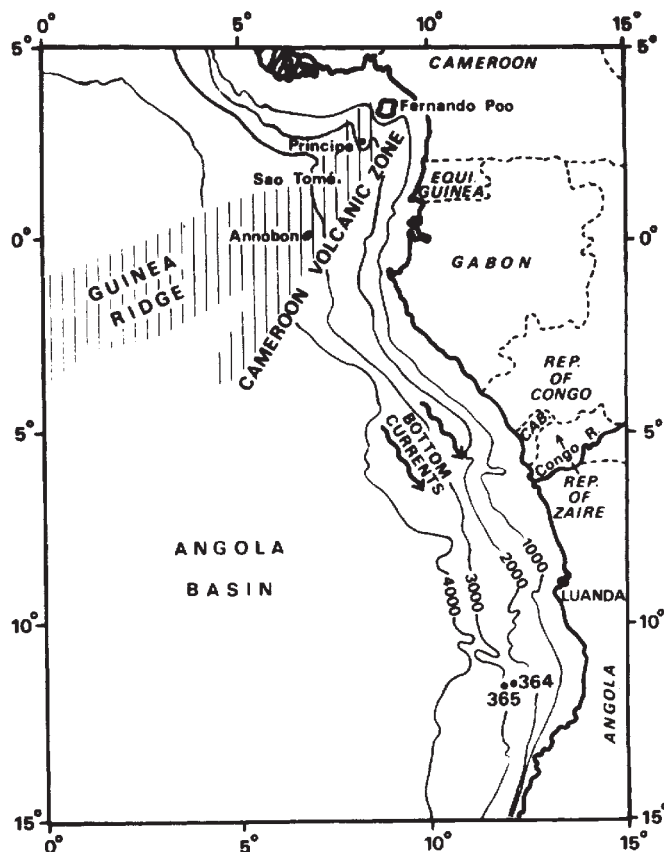
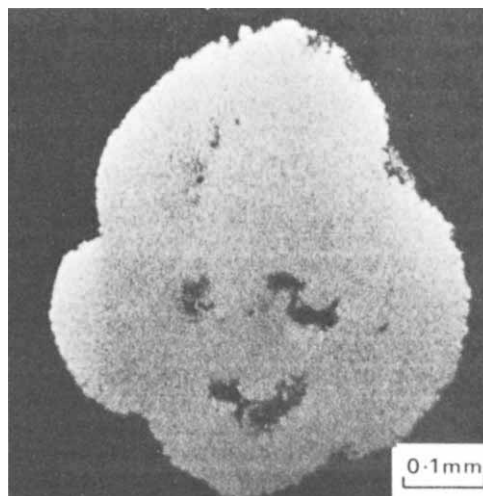


Fig. 1 Location map after Emery *et al.*¹⁶ showing site 364, where native copper has been found, and site 365. Isobaths in metres.

Syngenetic origin of copper in marine sediments requires: first, a source of the metal; second, transporting solutions and a force to move them and third, a precipitating agent. Several conceivable origins for the metal: a deep-seated magma, with ascending hydrothermal solutions carrying copper, extraction and concentration of copper from seawater by microorganisms, whose death causes localised enrichment of copper in the sediments (for example, the Kupferschiefer Formation⁴⁻⁶), or, finally, copper solutions derived from a submarine basaltic extrusion and transported to the site by currents.

Fig. 2 Electron microprobe scan showing homogeneous distribution of copper in a planktonic foraminifer cast. All light areas are copper; dark areas within the cast are unfilled spaces.



Several lines of sedimentological and chemical evidence militate against the first two possibilities⁷, and the third possible source is the most attractive. This type of source has been described as "volcanic emanations" or "hydrothermal exhalations" by many authors. Geochemical evidence has been presented which would seem to prove the presence of submarine hydrothermal solutions⁸. The margins of hot, submarine-erupted basaltic rocks are quickly cooled, forming pillow structures whose chemistry approximates the chemistry of the erupted liquid. The slowly cooled interiors of the flows are, on the other hand, depleted in certain elements which are known to be enriched in pelagic sediments and manganese nodules⁸. This depletion is caused by seawater entering the eruptive flow through contraction fractures, being heated, and dissolving volatiles and metals concentrated in the residual phases within the interior of the extrusion. Metals are then mobilised as chloride complexes⁸. This is in accord with work showing that a Na-Ca-Cl brine is a potent solvent for copper and other metals⁹.

This heavy, metal-enriched brine could be expected to flow over the sea floor, directed by gravity, bottom currents, or both. It is tempting to consider the north-east-south-west trending Cameroon Volcanic Zone as the possible extrusive source. This volcanic zone extends almost 600 nautical miles offshore, the terminal end being <700 nautical miles north of site 364 (Fig. 1). Moreover, the offshore segment is known to have extruded basalt during the Miocene⁹. Present-day movement of bottom currents in the eastern Angola Basin is to the south¹⁰. If a similar circulation prevailed during the Miocene—and it is believed that Neogene current patterns were similar to those of today¹¹—transport of the brine to the site 364 area would have been the result.

No matter where a copper solution came from, or how it eventually reached site 364, some agency caused it to precipitate native copper. Organic matter has been suggested as a possible agent^{12,3}. There have been several reports of native copper forming in modern peat swamps rich in organic matter^{12–14}. This is especially intriguing in view of the occurrence of authigenic marcasite at site 364 (ref. 15). Marcasite is a

other metals from basalt, if this were the source. Perhaps the fact that the common metals precipitate (as sulphides) in the order Cu, Zn, Pb, Ag may be relevant. Could copper have been precipitated here, early and alone, then the brine transported elsewhere to deposit other metals?

The foregoing discussion of the origin of this copper is highly speculative; it would be naive to think that a final and complete answer can be given yet. Nevertheless, it is hoped that the hypotheses presented will stimulate further ideas on the genesis of this unusual copper deposit.

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Table 1 Analysis of selected elements

Electron microprobe (%)			X-ray fluorescence (p.p.m.)	
	Foraminifera		Mass	< 89-μm fraction
	Specimen 1	Specimen 2		
Cu	99.92	99.08	99.83	Cu 3,029.38
S	0.18	—	0.64	Sr 556.20
Ca	0.08	—	0.01	Zn 204.75
Mg	0.05	—	0.01	Rb 95.46
Al	0.05	—	0.01	Ni 90.47
Fe	0.04	—	0.06	Zr 74.32
K	0.02	—	0.00	Y 20.62
Mn	0.01	—	0.01	Nb 6.92
Si	0.00	—	0.03	
Ti	0.00	—	0.01	
	100.35		100.61	

mineral normally associated with peat swamps and is unusual in the marine environment. Its sporadic occurrence at site 364 suggests periodic conditions of extreme acidity. Bacteria may assist organic matter in precipitating native copper in acidic swamps. Indeed, metallic copper has been produced experimentally from copper solutions by using swamp bacteria¹². Apparently native copper is formed by the reaction of bacterial waste products with copper solutions¹². A concentration of organic matter may have been the precipitating agent at site 364, even though the sediments are not enriched in organic matter today.

Many questions still remain unanswered. Why, for example, are no other metals or their sulphides (other than pyrite) found at this horizon? Surely hot brine would have dissolved

Experimental evidence that oxygen is the principal impurity in natural diamonds

In virtually all treatments of the physical properties of natural diamonds, nitrogen is considered to be a major impurity and is used to explain many properties (see ref. 1). This is true because of the analysis of ref. 2 where a good correlation between nitrogen concentration and optical spectra was found. Those results were obtained from the conversion of diamonds to graphite in a carbon crucible. The report that nitrogen is the major impurity in type I diamonds can be tested indirectly and qualitatively by analysing occluded gases released by crushing such diamonds. Numerous experiments in this laboratory on diamonds from Africa, North and South America have failed to show that nitrogen is the major impurity in natural diamonds^{3,4} and we decided to reinvestigate the composition of gases released by the conversion to graphite of natural diamonds. We found oxygen and hydrogen to be the major impurities, and nitrogen to have only a minor role.

The diamonds used were both type I and type II (as characterised by ultraviolet absorption⁵), and free of detectable inclusions under binocular microscope examination at 90×. Their body colour ranged from colourless to pale yellow and their weight ranged from 0.070 to 0.200 g. Possible contamination of the gases released from the surface of the apparatus was minimised by transforming the diamond crystals in a resistance-heated rhenium filament located near the centre of a large Nonex glass vacuum chamber. The vacuum chamber was attached to the inlet system of a research mass spectrometer³. Before being converted to graphite the diamond crystals were maintained at a temperature of 800 °C and a vacuum of 10⁻⁸ mmHg for several hours to remove possible surface contamination.

Table 1 Analysis of gas released by the conversion under vacuum of a natural type I diamond (0.143 g) to graphite

Gas T(°C)	1,500	1,800	1,900	Percentage abundance (volume)			
				2,000	2,200	2,700	2,900
H ₂	8.69	7.60	6.53	3.20	2.42	7.41	4.65
CH ₄	0.46	0.22	0.27	0.16	0.26	0.27	0.14
H ₂ O	0.89	3.03	9.90	4.15	2.39	3.02	5.12
CO	78.98	87.12	79.57	90.67	90.88	84.68	86.09
N ₂	1.05	0.86	0.90	0.78	0.82	0.73	0.79
Ar	0.03	0.02	0.02	0.02	0.02	0.01	0.02
CO ₂	9.90	1.15	2.81	1.02	3.21	3.88	3.19
Volume* (cm ³ STP)	0.05	0.06	0.06	0.07	0.05	0.03	0.01

*These volumes correspond to the following average weight % of atoms: H = 10.0%, C = 28.1%, N = 2.5%, O = 59.2% and Ar = 0.2%.

The several natural diamonds, both type I and type II from Arkansas, USA used, gave relatively consistent results. Analytical data from a typical experiment on a type I diamond are given in Table 1. The gases released from the diamond, in decreasing order of abundance, were CO, H₂, H₂O, CO₂, N₂, CH₄ and Ar. The average atomic weight % is O=59.2, C=28.1%, H=10%, N=2.5% and Ar=0.2%. These data are consistent with the gases released by the crushing of numerous other diamonds *in vacuo*^{3,4}.

To determine the amount of contamination derived from the apparatus, two different approaches were used: first, control experiments with no diamonds present were carried out to determine the amount of gas released from the apparatus and second several diamond crystals were converted to graphite at lower temperatures using platinum-rhodium filaments. These experiments showed that the maximum level of contamination for nitrogen was < 0.1% and that for other gases < 1%.

Since Kaiser and Bond⁵ used graphite in their experiments, we conducted a control experiment with graphite. Gas release from a preconditioned sample of spectrographic grade of graphite at 2,000 °C showed nitrogen to be the major component.

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cule have been observed. In Table 1 we present a Deslandres array based on data given by Krupenie². The entries underlined correspond to features which are clearly present in published nightglow spectra⁴⁻⁷. In particular the unexplained features pointed out by Chamberlain⁴ and Hennes⁶ are accounted for. The bands fall near a Condon locus which corresponds closely to that found for the Herzberg I system⁸ ($^3\Sigma^+_u \rightarrow ^3\Sigma^-_g$), where the Franck-Condon factors will be similar. Other bands of the $^3\Sigma^+_u \rightarrow ^1\Delta_g$ system may well be present in the nightglow, but are unclear because they coincide with bands of the Herzberg I system, or because they are weak. Such possible identifications are underlined with dashed lines.

As in the case of the Herzberg I system, there are a number of coincidences and near coincidences in wavelength of band origins. This arises because the vibrational quanta of the $^1\Delta_g$ state are approximately twice as large as those of the $^3\Sigma^+_u$ state. In cases where two or more bands might be present on wavelength considerations alone, comparison with bands present in the Herzberg I system⁸ provides a useful guide. Thus, for example, we identify the feature at 3,789 Å (refs 4 and 6) with the (3,2) band rather than the (1,1) band, although both are predicted to have an origin at 3,786 Å. Also, preferential population of the upper vibrational levels of $^3\Sigma^+_u$ is characteristic of the nightglow⁹. It is possible that emission from lower vibrational levels of $^3\Sigma^+_u$ to $^1\Delta_g$ is present in the nightglow, and contributes to the weakly banded blue pseudocontinuum⁹, but identification is not possible.

Laboratory investigations of the radiation emitted by recombination of atomic oxygen¹⁰ have shown the presence of numerous features between 3,500 Å and 5,000 Å which do not belong to the Herzberg I system. Degen¹¹ has presented a high resolution spectrum of a band with its origin near 4,007 Å, and stated that most of the other unidentified bands seem to belong to this system. The rotational structure is complicated,

Table 1 Calculated band origins in Å for the system $^3\Sigma^+_u \rightarrow ^1\Delta_g$ in O₂

v'' v'	0	1	2	3	4	5
0	3,687	3,900	4,135	4,395	4,685	5,008
1	3,584	3,786	4,007	4,251	4,521	4,822
2	3,491	3,682	3,891	<u>4,120</u>	<u>4,374</u>	4,655
3	3,407	<u>3,588</u>	<u>3,786</u>	<u>4,003</u>	<u>4,242</u>	4,506
4	3,330	<u>3,503</u>	<u>3,692</u>	<u>3,898</u>	<u>4,124</u>	4,373
5	3,261	<u>3,427</u>	<u>3,607</u>	<u>3,803</u>	4,018	4,254
6	3,199	<u>3,359</u>	<u>3,531</u>	<u>3,719</u>	3,925	4,150
7	3,144	<u>3,298</u>	<u>3,464</u>	<u>3,645</u>	3,842	4,057

Entries fully underlined seem definitely present in the nightglow; those partially underlined are probably present. Entries for $v' > 7$ are omitted because these levels of $^3\Sigma^+_u$ are believed to be absent in the upper atmosphere at night.

Nightglow and a new band system in molecular oxygen

I PROPOSE here that the transition $^3\Sigma^+_u \rightarrow ^1\Delta_g$ in O₂ is responsible for previously unidentified features in the nightglow from the Earth's atmosphere, and in observations of radiation emitted from recombining atomic oxygen in the laboratory. The transition violates the approximate selection rules $\Delta S = 0$ and $\Delta \Lambda = 0, \pm 1$ (ref. 1); it may be compared with the transition $^1\Delta_g \rightarrow ^3\Sigma^-_g$, which, in addition to violating these selection rules, also violates the rigorous selection rule $g \leftarrow / \rightarrow g$ for electric dipole radiation, and so proceeds by magnetic dipole radiation². The transition $^3\Sigma^+_u \rightarrow ^1\Delta_g$ may therefore be significantly stronger than the transition $^1\Delta_g \rightarrow ^3\Sigma^-_g$.

An accurate calculation of the wavelengths of the origins of the various bands of the $^3\Sigma^+_u \rightarrow ^1\Delta_g$ system is not possible, since only the lowest two vibrational levels of the free $^1\Delta_g$ mole-

as is to be expected for the $^3\Sigma_u^+ \rightarrow ^1\Delta_g$ system, which, like the $^1\Delta_g \rightarrow ^3\Sigma_g^-$ system, will have nine branches, three Q-form, two P-form, two R-form, one O-form, and one S-form (ref. 1). In general the calculated band origins in Table 1 are a few Å less than the band positions observed in the nightglow and in the laboratory. Thus we identify Degen's band at 4,007 Å with the (3,3) band. This may be partly because the bands are red degraded, and the levels corresponding to $J=0$ and 1 in $^1\Delta_g$ are missing, and partly because the vibrational terms for $v > 1$ in $^1\Delta_g$ need revising. A successful analysis of the rotational structure observed by Degen in terms of the transition $^3\Sigma_u^+ \rightarrow ^1\Delta_g$ would not only confirm this identification but also give accurate values for the higher vibrational terms of $^1\Delta_g$. The hypothesis presented here seems superior to the proposal of Chamberlain¹² that some of these features are due to the $^3\Delta_u \rightarrow ^1\Delta_g$ transition in O_2 ; not only do the wavelengths seem to agree better, but the $^3\Sigma_u^+$ state is already known to be present in laboratory systems and in the upper atmosphere at night. The contribution of either process to the population of the $^1\Delta_g$ state observed in the nightglow will be negligible.

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Imagery, affective arousal and memory consolidation

A DISPARITY exists between the methods and theory that have been developed to study human memory and those used in animal learning studies. Present research strategies in human memory focus on (1) differences in the stimuli-to-be stored in memory (such as the extent to which words can be encoded)¹; (2) conditions that precede new learning which influence the acquisition, stimulus transformation and encoding of input; (3) the context in which information is presented at the time of storage or retrieval from memory (where context determines the specific encoding strategy used to store and retrieve information)²; and (4) conditions that affect storage decay (such as those which might prevent rehearsal or precipitate “unlearning” of information stored in memory). The main theme of this research stresses

stimulus characteristics, often causally defined, as determinants of input-output relationships in memory. Few attempts have been made to develop the structural detail of human memory with respect to underlying biological mechanisms.

Studies of biological determinants and mechanisms of learning and memory have generally been on animals and have focused on neurochemical and neurophysiological conditions that alter learning or retrieval. Unlike human learning studies, these have involved physiological manipulations of brain after learning has taken place, at a time when experience is thought to be consolidated in memory. This research and its developing theory have emphasised the role of time- and intensity-dependent, specific and non-specific, brain state arousal in memory consolidation³. Unfortunately, few bridges exist between the methods and theory used in the biologically oriented animal learning studies and those thought useful in researching human learning. In this study we have attempted to integrate these two territories of findings and theory through manipulations of stimulus imagery and consequent encodability and of arousal using measures which in separate studies and approaches reliably affect recall probability.

Forty college students were first practised and then individually tested in a control condition and one of four experimental conditions. In the control condition, subjects viewed eight different groups of four common English words, each group consisting of two easily and vividly imageable words and two words difficult to image⁴. After viewing each word group for 4 s, subjects were asked to produce a single representative word which would serve to integrate each cluster of four words and to rehearse, or think about, the to-be-remembered words, for 10 s. After the subjects had seen and rehearsed all of the 32 words, they were engaged in a non-verbal distracting task for 10 min and then attempted to recall the presented words.

Different groups of these same subjects, ten in each, were also tested under one of four experimental conditions, constituting an imagery mnemonic and/or affective arousal manipulation superimposed on the control procedure. In all of these experimental conditions, subjects were provided with pictures projected from slides which were designed to help them organise input and aid recall. Pictorial stimuli depicted common human interactive themes, and were standard but ambiguous stimuli with known response characteristics [Thematic Apperception Test (TAT) plates 1, 3BM, 4, 5, 6BM, 7, 11, 13MF] which are used clinically to elicit emotionally rich themes as a projective personality test. In two of the conditions, these stimuli were presented for 10 s before the 4-s presentation of each cluster of four to-be-remembered words. The two remaining conditions involved presenting word clusters first (4 s) followed by the 10-s exposure of these same TAT slides during the time that to-be-remembered words were being rehearsed in immediate memory. Finally, one of each of the above two groups viewed the TAT slides under an instructional set to “get

Table 1 Free recall of words as a function of the timing of imagery mnemonics and affective arousal experiment conditions

		T ₁	T ₂	T ₁ A	T ₂ A
Mean total word recall	HI	6.5±0.4	7.4±1.2	8.3±0.8	11.1±0.9
	LI	3.9±0.5	4.3±1.0	5.0±0.7	7.8±0.8
	W/C	1.8±0.1	2.2±0.1	2.2±0.1	2.6±0.2
Change in recall produced by experimental conditions compared with control performance	HI	1.6±0.7	0.5±0.9	1.0±0.8	3.7±1.3
	LI	1.3±0.6	1.0±0.8	1.2±1.0	3.8±0.9

Subjects were studied in the following conditions: a control involving word recall without the use of imagery mnemonics or affective arousal; four experimental conditions, two involving only the use of an imagery mnemonic before the presentation of to-be-remembered word clusters (T₁) or after the presentation of these four-word sets (T₂) and the remaining two conditions using the imagery mnemonic with the induction of affective involvement before the presentation of each word cluster, (T₁A) or after their presentation, during rehearsal (T₂A). HI, High imagery words; LI, low imagery words; W/C, average number of words per recalled cluster.

involved with the projected interpersonal theme, by thinking about your own personal, relevant experiences". This manipulated set had been pretested in an independent group of 20 subjects and was shown to produce significantly higher self-ratings along a continuous global scale⁴ of affective involvement compared to a set of eight non-projective test stimuli. These subjective ratings were also significantly greater than those produced in response to a content-equivalent set of conditions in which no such instructional set was introduced. At the same time, these two sets of stimuli produced the same ratings on a global imagery scale used to measure the effectiveness of the stimuli as an imagery mnemonic.

The contrasting effects of these conditions on components of recall were examined by means of a series of ANOVA. Results are displayed in Table 1 which describes changes in: (1) recall of high compared with low imagery words; (2) total recall of words; (3) the number of words per cluster that could be recalled (a measure reflecting within group structure or salience where words per cluster measures different memory processes than are involved in accessing a higher order memory unit, cluster recall²). The number of clusters of presented information recalled, where at least one item from that cluster was remembered, is a measure that reflects retrieval of higher order memory units and access to these structures stored in memory². The number of groups of words recalled is the quotient of total word recall and word per cluster recall.

Analysis of variance demonstrated the following patterns of statistically significant effects. Control conditions consistently produced lower total recall scores than the experimental conditions, an effect due to fewer clusters of words recalled rather than differences in words per recalled clusters ($F=9.62$; d.f.=4, 45; $P<0.01$). In all conditions, more high imagery than low imagery words were recalled ($F=7.63$; d.f.=1, 45; $P<0.01$). Apparently the use of the imagery mnemonic made both high and low imagery words more accessible in memory. Imagery seemed always to change the salience of the stored stimulus trace.

Affective arousal strongly facilitated recall when information was already in memory, during a period of rehearsal and consolidation ($F=8.14$, d.f. 3, 27; $P<0.01$). Affective arousal was not effective at the time of information storage itself. Post-storage arousal facilitated recall, but increased not the number of higher order memory units (clusters) which could be retrieved but the number of words recalled per cluster (intrastructure salience).

Generally, treatments used in typical human memory studies are effective in inducing changes in the recall of higher order memory units but have little effect on recall of elements within such structure. In spite of the changes in recall produced by imagery and post-storage arousal, retrieval strategies for accessing memory were not altered. For example, neither input serial position effects on recall were altered by any of the manipulations used here, nor was output sequencing or clustering, or the tendency to maintain the integrity of higher order memory units at time of retrieval, altered in control or experimental conditions.

Our findings on the effect of arousal on memory consolidation are consistent with those in animal studies in which arousal by foot shock or drugs after learning alters trace consolidation⁵, and also with findings in some clinical studies where patients with disturbances in affect, for example, depression, show disturbances in the consolidation phase of memory which is reversible by drugs that alter affect and arousal (for example, L-dopa which influences the dopamine system in brain)⁶. These findings and the experimental design that was used to test effects of the timing of the imagery mnemonic in association with affective arousal also points out the value of bridging the methods and theories common to traditional human memory studies, with their emphasis on storage effects, and

the relatively greater focus on post-learning variables commonly used in biologically oriented animal learning studies.

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Antagonism between visual channels for pattern and movement?

GEORGESON has reported¹ some ingenious psychophysical experiments designed to test the hypothesis that 'sustained' visual cortical cells sensitive to the orientation of contours are antagonistically coupled to 'transient' cells in the same cortical column sensitive to movement at right angles to those contours. After inspecting one or other, or both, of two counter-rotating dot patterns, his subjects reported seeing "complex patterns of radial lines or curves". These appeared either to rotate in the opposite sense to the stimulus, or to be stationary if both counter-rotating disks had been viewed simultaneously in superposition. Georgeson attributed their appearance to the rebound response of 'pattern channels' inhibited during stimulation by 'motion channels' of the kind presumed to be responsible for movement after effects (MAE). We have tested this conclusion by extending the range of stimulus velocities well above and below those used by Georgeson. Our results suggest that stimulation of motion channels is not necessary for the effect observed, so that its appearance in his tests does not confirm his hypothesis.

Our doubts arose because at the speed used by Georgeson (66 r.p.m.) the retinal image of his rotating dot patterns must have generated appreciable circumferential streaks; and these alone could be expected to give rise to a 'complementary image' of radial streamers^{2,3,5} without invoking stimulus movement as such. This point was first recognised by Wilson⁵, who in fact described the same phenomenon in the late 1950s, together with the method of using two counter-rotating textured fields as the inducing stimulus. He listed the following after effects as the speed of rotation of the superimposed fields was increased: "(1) A set of faint, fine concentric circles were observed. (2) These became fewer but stronger and broader. (3) They expanded even more, radial whiskers became visible on the outer circles, and a general impression of flicker was observed. (4) The circles were then seen to be entirely composed of fine radial 'whiskers' or 'sparks' and appeared to pulsate in diameter. (5) With further increase of speed the circles expanded (leaving) radii, or sometimes a lattice pattern of opposite spirals, filling the whole field."

Wilson noted that the induction of orthogonal after images by rapidly moving stimuli had been described in 1845 by Sir David Brewster⁶, who observed it after staring from a moving railway carriage. (Wohlgemuth, in his classical monograph *On the aftereffect of seen movement*⁴, tried to explain Brewster's statement that the after effect was "transverse" to the direction of the objective movement

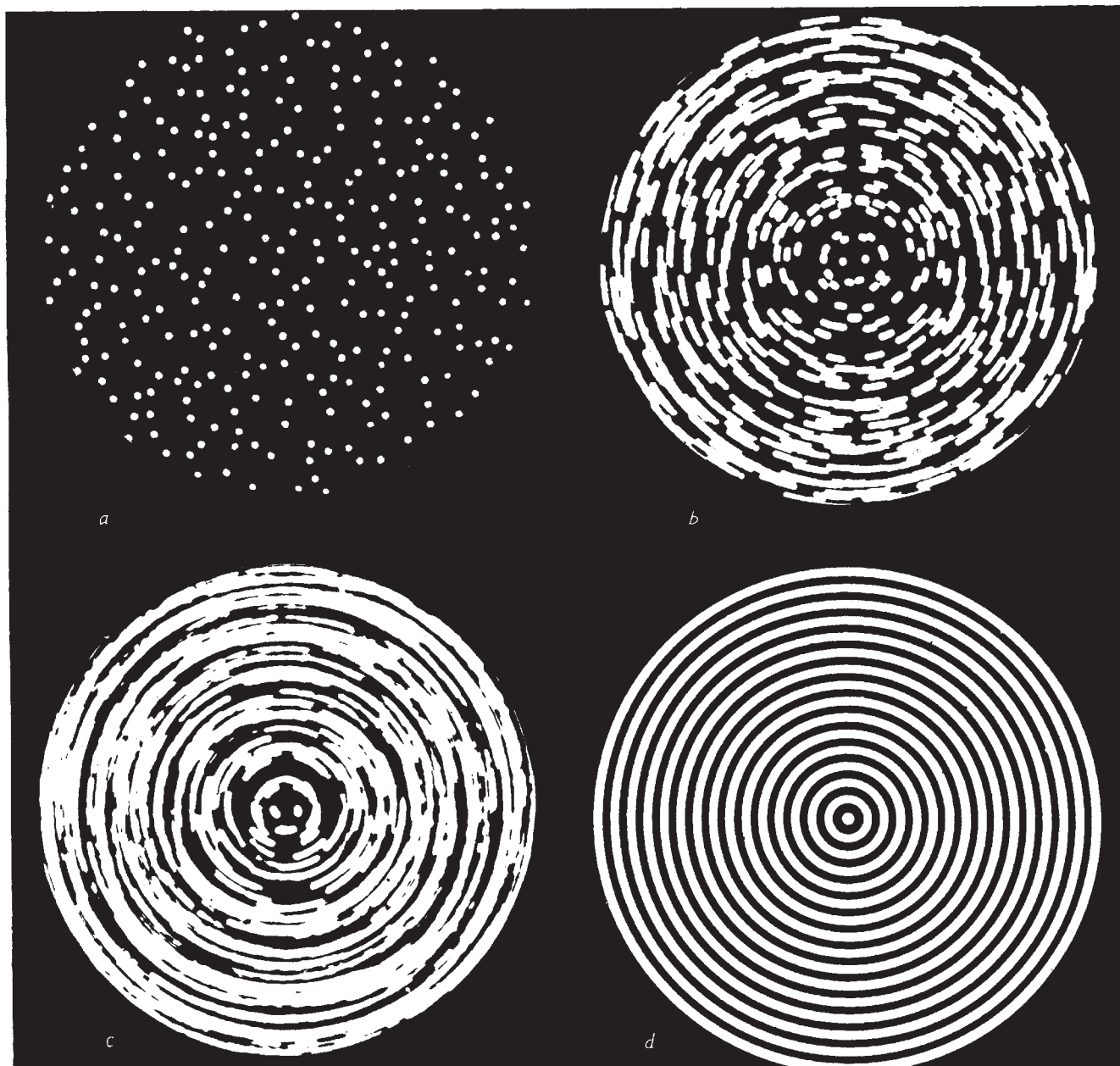


Fig. 1 *a*, Dot pattern used for counter-rotating stimuli; *b*, result of rotating *a* through 10° during exposure, and superposing mirror image; *c*, as *b* but 40° rotation; *d*, concentric-circle figure which excites a petaloid complementary image (see text).

as "possibly due to his changing the position of his head when he closed his eyes"!)) Wilson later reported⁷ that similar radial 'whiskers' can be induced by stroboscopic illumination of a single stationary circular contour.

Georgeson indicated that the 'movement analysers' he had in mind are those responsible for the familiar motion after effect or 'waterfall illusion'. Since the optimal rotation rate to generate a normal MAE is much lower than 66 r.p.m., human movement-sensitive channels should be more strongly stimulated if lower speeds were used. If Georgeson's interpretation were correct, this should intensify the radial lines seen as an after effect, and these might be expected to persist down to speeds at which the normal MAE disappears. Conversely, they should disappear at speeds above the upper threshold for perception of motion. If, however, radial lines were produced by the same mechanism as the normal complementary after image induced by inspection of stationary circular arcs (that is, by activation of contour-sensitive channels), they should persist at high rates of rotation, but disappear for rates that are too

low to produce significant circumferential streaks even though adequate for the normal MAE. We have therefore repeated Georgeson's observations, with eight subjects in all, using rates of stimulus rotation from 960 r.p.m. down to 3 r.p.m.

Our subjects viewed from 2.6 m a white screen on which two counter-rotating random-dot patterns were thrown from a 300-W projector having a motor-driven slide rotator. The two concentric images, produced with the aid of a Dove prism in front of the projector lens, were matched for brightness (110 cd m^{-2} on white parts). They subtended an angle of approximately 20° at the subject's eyes, and were centrally fixated.

After an exposure of 1 min the subject immediately inspected a uniform white surface (70 cd m^{-2}) and was asked to report any after images. For stimuli rotating at 60 r.p.m., all our subjects confirmed Georgeson's report of near-radial lines, usually adding that these seemed to "shimmer" or "stream" radially. Most subjects saw them extend almost to the centre, with only the central 4° clear of lines.

With a stimulus rotating at 30 r.p.m., however, six of seven subjects reported that the lines came only "about halfway in", leaving an 8° central area clear of radial lines. At 10 r.p.m. (which gave powerful MAEs with a single disk) only two of six subjects saw an after image of "faint radial whiskers" near the periphery of the stimulated field. At 6 r.p.m. all of seven subjects reported the whole test field free of radial lines, with only faint pulsating concentric rings as an after image, except that one subject (VM) detected faint radial "whiskers" around the periphery. Finally, three subjects tested at 3 r.p.m. could see no radial lines; yet the MAE at this rate (for a single disk) was still powerful over the whole area. As might be expected, the exact cutoff point for the radial after effect varies with individuals and with the rotating pattern used; but the trend of these results is clearly opposite to that predictable on Georgeson's interpretation.

Increasing the speed above Georgeson's rate of 66 r.p.m., we found no noticeable reduction in intensity of the chrysanthemum-like complementary after image up to 240 r.p.m. At 480 r.p.m. and 960 r.p.m., all observers agreed that it was considerably weaker, although still detectable; but at these speeds, the streaks produced by individual dots would be expected, and were observed, to run into each other and reduce effective contrast.

A rough quantitative check on this point can be gained by calculating the patterns of streaks to be expected if each retinal ganglion cell fired for 60 ms in response to a briefly flashed spot⁸. During this time at 60 r.p.m. each dot of a rotating pattern would trace an arc of about 20°. Figure 1a shows the dot pattern we used, and Fig. 1b and c the results of rotating it through 10° and 40° during photographic exposure and then superposing the mirror image, to simulate the streaks produced by counter-rotating fields at rates of 30 and 120 r.p.m. on the foregoing assumption. It is clear that (1) overlapping of streaks at these rates does not prevent a strongly circular pattern from developing; (2) at rates below 30 r.p.m. a progressively larger central area would become practically streak-free, and so ineffective in stimulating contour analysers; and (3) at rates above 120 r.p.m. (for our dot pattern) overlapping of streaks must progressively reduce effective contrast. This all fits quite well with the observations, and suggests that the assumed duration is of the right order of magnitude.

With a moving dot, the stimulating streak is effectively pulsed on and off as the dot passes over each receptive field, so the after effect would be expected to be (and is) stronger than that after inspecting Fig. 1b and c. Figure 1d (see ref. 2) has been included to let the reader verify that the complementary image induced by stationary concentric circles (or by a line of dots rotating at very high speed) is indeed an array of near-radial streamers. These may be seen either immediately on closing the eyes or on a uniform white surface. (At repetition rates of the order of 5–10 s⁻¹, radial streamers can be seen continuously during inspection. (Compare ref. 3, page 344.)

We conclude that Wilson's radial after effect is most parsimoniously attributed to stimulation of contour analysers by the rotating dots, and that stimulation of the motion analysers responsible for the MAE is not essential. This does not of course disprove the general idea of opponent coupling between 'sustained' and 'transient' cells. It was shown² that similar complementary images can be made visible continuously (not only as an after effect) by optically superimposing dynamic visual noise (for example, from a detuned TV receiver) on the fields of near-parallel contours, such as Fig. 1d^{2,9}. The inference then suggested (before the discovery of contour-specific visual cells) was that channels sensitive to one orientation are rendered differentially hyperexcitable by 'satiation' of channels sensitive to the orthogonal orientation. Whether the

mechanism was one of active mutual inhibition or of passive competition was left open by the psychophysical evidence; but in view of Wilson's finding⁵ that the full effect requires the presence of about four to six near-parallel lines, and the fact that the wavelength of the sinuous illusory contours is typically about 4 times the inducing linespacing, it seems likely that network properties as well as single-unit characteristics are involved^{2,3}. Georgeson's suggestion of antagonism between pattern- and motion-signalling channels is geared to the idea that removal of the parallel-line inducing pattern 'disinhibits' motion channels. The orthogonal patterns seen in dynamic noise suggest that even in the presence of the inducing lines, orthogonal channels for both pattern and motion may become hyperexcitable. In any case, we agree with Georgeson that it seems attractive to invoke excitation of motion channels in order to account for the streaming motion observed in the after effect, even if more complex relationships must be added to the picture to do justice to all the facts.

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Evidence for a low upper limit of heritability of mental test performance in a national sample of twins

THE use of kinship correlations is a long established approach to the estimation of heritabilities (h^2) and component variances of human phenotypes. Among the best known studies are those using monozygotic and dizygotic twin correlations, data from which are commonly interpreted to represent a substantial heritability (that is, about 0.8) of IQ and other mental test performances (see Jensen¹ for review, and Jinks and Fulker² for the empirical requirements of this approach). Some doubt has been cast, however, over the empirical sufficiency of the most quoted estimates. Among the doubts are those concerning biographies of subjects, testing procedures, and problems emerging from the use of varied and inappropriate tests and from the subjects being of widely different ages³. More recent estimates using a version of the identical-fraternal twin comparisons (but derived from a sample in which the zygosity was unknown) have arrived at a value for h^2 not significantly different from zero^{4–6}. The National Child Development Study (for details, see ref. 7), a longitudinal study of all the children in England, Scotland and Wales born in one week of March 1958, includes a nationally representative sample of twins about whom have been gathered considerable biographic, biometric, social and psychometric data (unpublished results). We have used this sample to investigate the broad heritability of performance on a general mental ability test.

Zygosity of twins were established during an interview of parents of twins by health visitors at the 7-yr follow-up and from responses to a letter sent to parents at the time of the

Table 1 Means ($\pm$ s.d.) of verbal (V) and non-verbal (NV) test scores by zygosity and sex

	MZ		DZ _{ss}		DZ _{os}		Whole cohort	
	V	NV	V	NV	V	NV	V	NV
Male	19.857 $\pm$ 9.100 <i>n</i> = 42	20.405 $\pm$ 8.619	18.983 $\pm$ 8.865 <i>n</i> = 60	19.367 $\pm$ 6.978	17.750 $\pm$ 10.401 <i>n</i> = 40	19.050 $\pm$ 8.115	21.044 $\pm$ 9.466 <i>n</i> = 7,262	20.754 $\pm$ 7.681
Female	18.825 $\pm$ 8.743 <i>n</i> = 40	19.400 $\pm$ 6.719	22.300 $\pm$ 8.339 <i>n</i> = 50	21.360 $\pm$ 7.016	20.175 $\pm$ 7.576 <i>n</i> = 40	19.725 $\pm$ 7.791	23.124 $\pm$ 9.128 <i>n</i> = 6,872	21.015 $\pm$ 7.536
<i>t</i> =	0.392 (NS)	0.484 (NS)	1.579 (NS)	1.174 (NS)	1.625 (NS)	0.526 (NS)	13.285; <i>P</i> < 0.001	2.038 <i>P</i> < 0.05

NS, Not significant.

11-yr follow-up; they were asked if the twins were 'identical' or 'non-identical' that is, 'fraternal'. This does not, of course, provide conclusive evidence of zygosity; however, several studies have shown that simple questionnaire responses of this sort reach at least 95% agreement with serological criteria, and it is generally felt that misclassifications by this method are uncommon⁸⁻¹⁰. The proportions of identical and fraternal twins classified in this way in the present sample approximate the theoretical values, and there were a few pairs, excluded from the present study, for whom the zygosity was unknown.

comparisons. The differences are 0.185 for the verbal test (that is, appreciably greater than MZ-DZ_{ss}) and 0.117 for the non-verbal test. If we are correct in assuming only marginal sex bias in the test itself they probably reflect what is known as 'treatment effect', that is, inflation of the DZ_{ss} correlation by virtue of parents, teachers, peers and so on treating same-sex twins in a similar way. Identical twins, as well as necessarily being of the same sex, also look alike and have other characteristics in common. Not surprisingly, it has repeatedly been demonstrated that identical twins are generally subject to more

Table 2 Analysis of variance tables for verbal and non-verbal test scores

	MZ <i>n</i> = 41 pairs		DZ _{ss} <i>n</i> = 55 pairs		DZ _{os} <i>n</i> = 40 pairs		DZ _t <i>n</i> = 95 pairs	
	V	NV	V	NV	V	NV	V	NV
MS within pairs	12.207	7.335	13.118	9.773	22.569	16.569	17.097	12.634
MS between pairs	69.446	54.296	64.019	39.552	63.255	47.999	65.705	49.573
Intraclass correlation	0.701	0.762	0.659	0.604	0.474	0.487	0.587	0.594

The data considered here relate to the second follow-up study when the subjects were 11 yr old. At that time all the subjects were administered a timed (30-min), group test of general mental ability by their teachers in schools, following strict but simple instructions concerning timing and test circumstances. The test, comprising 80 alternate verbal and non-verbal items, was specially designed for survey work with children of this age group by the National Foundation for Educational Research (for further details of reliabilities and validities, see ref. 11). Although it has not been cross validated with better known standardised tests, the two sets of items comprising it would seem to be very similar to types used in typical IQ tests (Fig. 1). It is as well to draw attention to this because reported h^2 estimates for performance on scholastic attainment tests are often appreciably lower than those for general ability tests, although they vary widely depending on the precise nature of the test¹². Because of this we have calculated separate correlations for the verbal and non-verbal performances.

Table 1 shows the mean scores and standard deviations for the whole cohort and for twins separated by zygosity and sex. (Suffixes ss and os signify same sex and opposite sex, respectively.) Overall these figures suggest a marginally better performance for girls relative to boys, including those in the dizygotic (DZ) groups. This may be due to a sex bias in the test, the main consequence of which would be to reduce the DZ_{os} correlations slightly. Table 2 contains analysis of variance tables for verbal and non-verbal scores of twin pairs. A major question is the magnitude and statistical significance of difference between correlations. The most important ones from the point of view of heritability are (monozygotic) MZ-DZ_{ss}. These are 0.042 for verbal scores and 0.158 for non-verbal scores, neither of which is statistically significant. Such non-significant differences are in themselves supportive evidence for very low or zero heritabilities.

The differences between fraternal twins of the same and opposite sex (DZ_{ss}-DZ_{os}) are interesting because they suggest a possible source of error in identical-fraternal twin

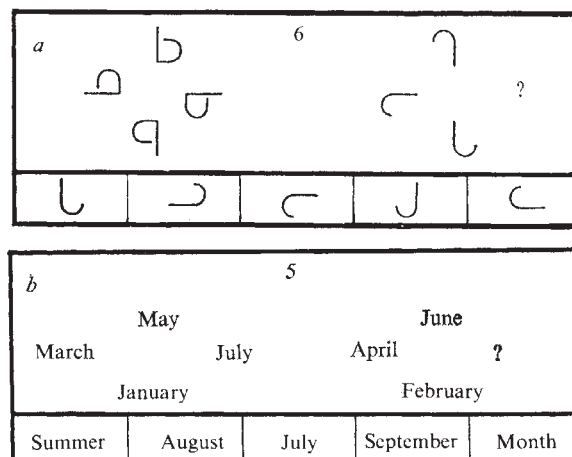
uniform treatment than fraternal^{5,6}. It is therefore possible to invoke treatment effects as the basis for inflated MZ twin correlations.

Table 2 indeed illustrates what happens when these 'treatment effects' are incorporated into the estimation of heritability (t indicates the total obtained from both os and ss twins). The difference MZ-DZ_t is non-significant for the verbal test, but although remaining small, becomes significant for the non-verbal test; using Z scores we have

$$Z_{MZ} = 1.000; Z_{DZt} = 0.683$$

$$\frac{\Delta Z(MZ-DZt)}{\sigma(Z_{MZ}-Z_{DZt})} = \frac{0.317}{0.190} = 1.668 (P < 0.05)$$

On the face of it these data yield a heritability of 0.336 ± 0.189

Fig. 1 Examples of (a) non-verbal and (b) verbal items used in the mental ability test.

for non-verbal mental ability. This is obtained from the general expression, $h^2 = (r_{MZ} - r_{DZ})/(\rho_{MZ} - \rho_{DZ})$, where ρ_{MZ} and ρ_{DZ} are the theoretical correlations for identical and fraternal twins, respectively; essentially the derivation of Jensen¹². Taking $\rho_{DZ} = 0.5$, of course, assumes no assortative mating (the presence of which would increase h^2) and no dominance or epistasis (which would decrease h^2). The magnitude of these influences is difficult to assess with accuracy from existing empirical data, but it is usually assumed that they are very small. Nonetheless, adopting Jensens¹² estimate of $\rho_{DZ} = 0.55$ under assortative mating, and thus the maximum feasible correction to the above h^2 , only increases it to 0.373. This h^2 is the consequence of introducing the usually unsuspected 'treatment effects' into the data and is at best an upper limit. It has been demonstrated that taking only minimal account of such effects in the data of previous studies reduces the reported h^2 estimates to values not significantly different from zero^{5,6}.

In conclusion, our nationally representative sample of twins of uniform age and test subjection, offer supportive evidence for zero or low upper limit heritabilities of mental test performance. Such estimates are considerably lower than the value of 0.8 normally quoted in psychometric research¹³ and which has permeated the psychological literature. Although it is necessary to be clear about the often-inflated significance of heritability estimates¹⁴, the present results are perhaps further indicative of the stringencies of sampling required in such studies³.

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Correction to Fisher's correlations between relatives and environmental effects

FISHER¹ has given a now much used model of assortative mating, and using formulae obtained for it he analysed data² on human height, span and forearm. In his model, phenotypic variance is regarded as the sum of three variances, additive, dominance and environmental; and genetic variance is the sum of the additive and dominance components. This was Fisher's first important contribution to genetics and it would seem that he did not appreciate fully the implications of his model. His formulae for parent-child correlation and sib correlation, in the presence of assortative mating, are not correct for his model. Moreover, the correct formulae for these correlations for his model suggest a different interpretation when the value for (genetic/phenotypic variance) is greater than 1. I present here a summary of criticisms of Fisher's formulae; details of which, with derivation of correct formulae will be presented elsewhere.

The additive variance is obtained by fitting a linear regression on the gene content of the genotype; dominance variance is the residual genetic variance, that is, genetic, additive variance. Fisher found that the value of his symbol c_1 defined as (genetic variance/phenotypic variance) was greater than 1 for height and span and concluded that this "gives no support to the supposition that there is any cause of variance in these growth features other than genetic differences". His method of partitioning the phenotypic variance into additive, dominance and other components has recently been used for IQ and estimates of its heritability (additive variance/phenotypic variance) have been the subject of some controversy.

Fisher realised that assortative mating will cause association between genotypes ("phases of factors" in Fisher's terminology). He devised a complicated method to take account of it and found that this association increases the genetic variance of the population. The increase in genetic variance is attributable entirely to an increase in the additive variance of the population³.

Fisher did not consider what effect assortative mating has on the genotypes of a parent and his progeny. He assumed that the additive deviations are the only cause of resemblance between parent and child as he says, "Hence, since there is no association except of z (additive values) between parent and child, the parental correlation coefficient is

$$c_1 c_2 \frac{1 \times \mu}{2}$$

Here μ is the phenotypic correlation between husband and wife and c_2 is the total additive variance/genotypic variance; c_1 has already been defined.

Wright⁴ on the other hand says that "Assortative mating introduces a correlation between dominance deviations of parents and offspring and between dominance deviations of either and additive deviations of the other". Fisher did not investigate such correlations and took no account of them. It can be shown that in Fisher's model of assortative mating there is a small correlation between the additive and dominance deviations of parent and child. Thus, the assumption on which Fisher obtained his formula for parent-child correlation is not correct.

To obtain his formula for sib correlation, Fisher discarded his model of assortative mating and reverted to random mating. He said, "The variance of a sibship, for example, depends, apart from environment, only on the number of factors in which the parents are heterozygous, and since the proportion of heterozygotes is only diminished by a quantity of the second order, the mean variance of the sibships must be taken for our purposes to have the value appropriate to random mating. . . ."

It is true that the variance of single sibship will be the same under random mating and assortative mating. The proportions of different types of matings will, however, be different under the two systems. As the mean sibship variance is $\Sigma(\text{frequency} \times \text{sibship variance})$, it will not be the same under the two systems.

Moreover, Fisher adds the whole of the increase in variance, $A\tau^2/(1-A)$ where A is the genetic correlation between husband and wife and τ^2 is the additive variance appropriate to random mating, to sib covariance and no part of the increase to sib variance. This is difficult to justify assuming Mendelian segregation on which his model is based.

Consider now his two formulae together

$$\begin{aligned}\text{Parent-child correlation} &= \frac{1}{2}c_1c_2(1 + \mu) \\ \text{Sib correlation} &= \frac{1}{4}c_1(1 + c_2 + 2c_2A)\end{aligned}$$

In the presence of dominance, the sib correlation will be greater than the parent-child correlation, therefore

$$\frac{1}{4}c_1(1 + c_2 + 2c_2A) > \frac{1}{2}c_1c_2(1 + \mu)$$

which holds only if $(1 - c_2)/2c_2\mu > 1 - c_1c_2$. Fisher's model of

assortative mating will hold for those values of c_1, c_2 and μ for which this inequality holds. It can be verified that it holds only for certain combinations of values of these parameters. For example, if $\mu = 0.5$, $c_1 = 0.8$, $c_2 = 0.8$, we get $0.25 > 0.36$, which is not true.

These considerations lead to the conclusion that Fisher's formulae for parent-child and sib correlations are not correct for his model. It is, however, possible to obtain formulae using Fisher's model which do not suffer from these deficiencies. These formulae, in Fisher's notation, are

Parent-child

$$\text{correlation} = \frac{1}{2}c_1c_2[1 + A(1-A)^2] + \frac{1}{2}c_1(1-c_2)A(1-A)$$

Sib correlation = $\frac{1}{2}c_1c_2[1 + A(1-A)^2] + \frac{1}{4}c_1(1-c_2)$

In both, the first term represents the contribution of additive deviations to correlation and the second term the contribution of dominance deviations. Note that the contribution of the dominance deviations to parent-child correlation is smaller than their contribution to the sib correlation since $A(1-A) \leq \frac{1}{4}$. This is another reason why the concept of heritability defined as additive variance/phenotypic variance cannot be applied to human populations mating assortatively.

If the coefficient of assortative mating μ is known, the value of c_1 and c_2 can be obtained from the parent-child and the sib correlation formulae. Effectively, the difference between the two correlations gives a fraction of the dominance variance. The formulae given here and those obtained by Fisher, however, are for genetic correlations which are unknown and phenotypic correlations have been used instead. If the environments of sibs are more alike than those of parent and child, the difference between phenotypic correlations will also contain a fraction of the environmental variance. When the phenotypic correlational difference is too large to be explained by the amount of dominance variance in the population, the value of c_1 will come out to be greater than 1. Thus, such a value, far from being evidence for no environmental effects, indicates their presence. On the other hand, a value less than 1 for c_1 does not, of itself, exclude environmental effects, which could mean that the contributions of dominance deviations, as well as of environmental effects, are small.

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in developmental rates produced alterations in productivity, body size and viability of the flour beetles⁴. The reproductive fitness of chickens under selection for increased shank length was reduced steadily throughout the selection process⁵.

Lerner⁶ proposed that an increase in homozygosity is directly responsible for the reduction in reproductive fitness found in selected strains. He submitted evidence suggesting that heterozygosity is of prime importance in producing a balanced, highly fit natural population. This preselection phenotype is the outcome of numerous generations on which natural selection has operated to produce maximum reproductive fitness. As experimental selection proceeds, the balanced phenotype and genotype favoured by natural selection is disrupted. The selection process favours homozygosity for those genes which influence the selected trait. Homozygosity is also increased by incidental inbreeding, a consequence of the experimental selection procedure. On relaxation of the experimental selection pressures, natural selection is free to restore a balanced phenotype. The most heterozygous individuals are again favoured, causing the "gains" made in the experimental selection scheme to be lost. Lerner has called this phenomenon genetic homeostasis and defined it as "the property of the population to equilibrate its genetic composition and to resist sudden changes".

Selection for divergent geotactic and phototactic maze behaviour in *Drosophila* is also thought to decrease reproductive fitness. The evidence is indirect and based on the observation that suspension of selection pressures in strains of *D. pseudoobscura* resulted in the reversion of the positive and negative geotactic and phototactic strains towards their original neutral maze response⁷. No decrease in productivity has, however, been found in divergent geotactic strains of *D. melanogaster* during 10 generations of selection⁸. This present study was undertaken to investigate directly the reproductive fitness of strains of *D. melanogaster* selected for positive and negative geotactic and phototactic maze behaviour.

Fifteen-unit classification mazes^{9,10} were used to select experimentally for divergent geotactic and phototactic behaviour. Maze scores may vary from 1 to 16 on both geotactic and phototactic mazes. A score of 1 represents the most negative maze response possible and the maximum positive maze response results in a score of 16. A cage population of *D. melanogaster* which was established by hybridising large samples of 20 different wild-type strains, was used to begin selection for opposite geotactic and phototactic behaviour. Divergent behavioural selection was carried out by collecting approximately 300 females and 300 males which were run separately through either the geomazes or photo-mazes each generation. Sixty pairs of the most positive and negative individuals were selected as parents to initiate subsequent generations for both types of behaviour. Non-virgin females were run through the mazes so females selected as parents were desecrated by being placed at -10°C for 10 min¹¹. The geo-strains and photo-strains thus produced were highly divergent having undergone opposite selection pressures for over 40 generations. The degree of divergence is reflected in the mean geotactic and phototactic scores of the four strains. Following 42 generations of geotactic selection the mean score of the geonegative strain was 1.85 and the mean score of the geopositive strain was 14.90. The photonegative strain had a mean phototactic score of 2.01 while the mean score of the photopositive strain was 12.7. The unselected control strain was approximately neutral for both geotactic and phototactic behaviour throughout the selection process.

The following procedure was followed to estimate the egg-to-adult survival, egg hatchability, larva-to-adult survival and female fecundity of the strains under study. Virgin females and males were collected over a 48-h

Effects of artificial selection on reproductive fitness in *Drosophila*

A COMMON feature of many selection experiments, when polygenically determined traits are involved, is a reduction in the reproductive fitness of the selected strains. Experimental selection for differences in the numbers of abdominal bristles and sternopleural chaetae in *Drosophila melanogaster* produced sterility and a reduction in fertility in the selected strains^{1,2}. Latter³ found that the "competitive index" (mating propensity, female fecundity and survival ability) had fallen sharply in strains of *D. melanogaster* subjected to experimental selection for differences in scutellar bristle number. Selection in *Tribolium* for changes

Table 1 Egg-to-adult survival, egg hatchability and larva-to-adult survival of flies from the geotactic strains and phototactic strains

Strain	Mean % egg-to- adult survival	Mean % of eggs hatching	Mean % larva-to- adult survival (uncrowded)	Mean % larva-to- adult survival (crowded)
Geopositive				
Generation 42	56.6			
Generation 46				
Replication 1	57.6	81.9	91.4	70.0
Replication 2	58.0			
Geonegative				
Generation 42	74.2			
Generation 46				
Replication 1	68.9	92.7	88.8	76.4
Replication 2	69.3			
Photopositive				
Replication 1	61.6	87.6	92.2	74.1
Replication 2	68.2			
Photonegative				
Replication 1	65.2	86.8	88.7	78.6
Replication 2	71.2			
Control				
Tested at				
Generation 42	78.1			
Replication 1	72.8	91.7	96.3	83.5
Replication 2	78.7			

period and stored in separate half-pint food bottles. At the end of this period 25 pairs of adult flies (an average of 1 d old) were immobilised with ether and placed in an empty half-pint milk bottle. A food surface was placed on the open end of the milk bottle. Each food surface consisted of a Stender dish top filled with 5 ml of the standard medium covered with a circular piece of paper towelling coated with a thin suspension of bakers' yeast. The bottle was inverted after the flies' recovery from the ether treatment. At 12-h intervals thereafter, the bottle was turned right side up and the flies were gently knocked to the bottom of the bottle. The food surface was changed and the bottle was returned to the inverted position. The eggs that were laid during the preceding 12 h were then counted. The first 100 eggs counted on each surface for each 12-h period were transferred into a 37-ml shell food vial in groups of 10. This procedure was continued for 20 d as the females aged from 2 d to 21 d old. Approximately 13 d after each egg transfer the number of adults to eclose from each vial was recorded, giving a measure of egg-to-adult survival.

The number of eggs to hatch was estimated during the egg-to-adult survival experiment when 2 replications were tested for each strain. In this case, at 3-d intervals, the food surfaces were changed prematurely after 3 h. One hundred eggs from each strain were transferred on to a fresh food surface and the number of eggs to hatch was recorded 30 h later. A total of 700 eggs was transferred for each strain. The set of eggs produced during the following 9-h interval were used in the egg-to-adult survival experiment.

Larva-to-adult survival was estimated by transferring 50 first instar larvae recently hatched (from the above food surfaces) into food vials. The number of adults to eclose from these vials was recorded 13 d after the larvae transfer. A total of 350 larvae was transferred for each strain tested.

Initially, a single replication of the positive and negative geotactic strains (generation 42) was investigated for egg-to-adult survival and female fecundity. Subsequently, the egg-to-adult survival, number of eggs to hatch and larva-to-adult survival were measured for the phototactic, geotactic and control strains with 2 replications from each strain being tested for egg-to-adult survival. The egg-to-adult survival values (Table 1) were analysed by calculating a corrected sum of squares value for the replication

means within each strain. These values were summed over all strains and divided by 8, the total number of degrees of freedom, to approximate the variance of a replication mean. A *t* test (d.f.=8) was then used to compare the different paired replication means.

Table 1 reveals that egg-to-adult survival was significantly reduced in members of the geopositive strain compared with flies from the control strain ($t=5.70$, $P<0.01$) and the geonegative strain ($t=3.59$, $P<0.01$). The geonegative flies were not, however, different from the control flies ($t=2.11$, $P>0.05$). Individuals from both phototactic strains were significantly lower in survival value than the control strain flies (photopositive, $t=3.44$, $P<0.01$; photonegative, $t=2.40$, $P<0.05$) but they were not significantly different from each other for this fitness parameter ($t=1.05$, $0.5<P<0.3$).

The percentage of hatchings associated with the geopositive strain was also decreased by comparison with all other strains tested (Table 1). Little difference in larva-to-adult survival was found in the four experimental strains and the control strain. This suggests the decrease in egg-to-adult survival found in geopositive flies is likely due to decreased hatching success of their eggs. The egg-to-adult survival values are, however, on the average, only 0.8 as large as the products of the percentage of hatchings and larva-to-adult survival values. This probably stems from differences in the techniques involved in estimating these fitness parameters. For example, the larval density was greater in the egg-to-adult survival study which may have reduced larval viability and accentuated the between-strain differences, whereas in the larval transfer study the lower larval density resulted in more favourable conditions and larval viability may have been only minimally affected. In fact, by dividing the egg-to-adult survival values by the mean percentage of eggs hatching one can compute the average larval viability under crowded conditions (last column Table 1). This shows that the larval viability is reduced in all strains under the crowded conditions with the geopositive flies showing the lowest larval variability.

No differences were found in female fecundity among flies from the geotactic strains (generation 42) and the control strain. The mean number of eggs laid per female per d was 38.4 for the geopositive females, 34.6 for the geonegative females and 35.3 for the control females.

In summary, selection for divergent geotactic maze behaviour has reduced the egg-to-adult survival rate of the geopositive flies with no similar change observed in flies from the geonegative strain. This decrease in viability seemed to be the result of lowered egg hatching success and a decrease in larval viability in crowded cultures. A slight decrease was observed in egg-to-adult survival of both phototactic strains, but the photopositive flies were not different from the photonegative flies.

A decrease in reproductive fitness, as found in the phototactic flies, is not unexpected after experimental selection. The reduced egg-to-adult survival found in geopositive flies but not in geonegative flies is, however, surprising. What are the possible explanations for this differential decrease in reproductive fitness?

Latter and Robertson¹² also found an asymmetrical decrease in fitness in strains of *D. melanogaster* subjected to independent selection for 3 traits (abdominal bristle number, wing length and sternopleural bristle number). They observed that flies from the low selected strains were more reduced in reproductive fitness than flies from the high selected strains. It was concluded that some loci acted pleiotropically, influencing reproductive fitness and the selected phenotype. A reduction in fertility was noted by Wigan and Mather³ and Mather and Harrison¹ while selecting for chaeta number in strains of *D. melanogaster*. Decreases in fitness were found to accompany the initial generations of selection, but after selection pressures were

relaxed, the fitness of individuals from the selected strains was observed to increase. These results led to the hypothesis that genes influencing fitness were linked with those being directly selected. Selection favouring unique gene arrangements that control the morphological trait being selected was felt to disrupt concomitantly the fitness controlling genes. The increase in fitness after suspended selection pressures may represent crossover events which "release" the polygenes under direct selection from the fitness controlling genes.

In my own experiment an additional possible explanation is that the different selective pressures associated with geopositive and geonegative selection may be responsible for the asymmetrical decrease in fitness. The lack of egg-to-adult survival rate differences between the photonegative and photopositive flies supports this idea. The phototactic maze is horizontal and members of divergent phototactic strains would traverse the maze expending equivalent amounts of energy. Because geonegative selection favours flies that can walk through the entire vertical maze opposing the pull of gravity, those flies must also be the hardest and the most vigorous. Flies selected in the geopositive direction, in contrast, may include individuals that weakly stumble downwards, as well as those that walk purposefully down. Selection of the most energetic individuals in the geonegative direction may counteract the normally deleterious effects of experimental selection. While selection in both directions favours those individuals homozygous for geobehavioural genes, the concomitant selection for vigour in the negative direction may reduce the deleterious effects of inbreeding. King¹³ studied inbreeding in rats and demonstrated that selection for vigour does offset its fitness-reducing effects. Selection of weak and stumbling flies in the positive direction may have directly favoured the least fit and most homozygous individuals.

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A simple mechanism for population cycles

ALTHOUGH most animal populations exhibit irregular fluctuations, there has long been considerable interest in the dynamics of populations which seem to fluctuate in a cyclic manner with a period different from that of obvious environmental 'drivers'. We have analysed possible models of the mechanisms underlying the regulation of populations in order to simplify the extraction of useful information from specific cases of population oscillation.

Existing models of cycling populations involve one of three mechanisms:

- The population is assumed to be a component of a stable but underdamped system—that is, in the absence of environmental noise the system approaches equilibrium in a series of damped oscillations. Cyclic behaviour is interpreted as the response of such a system to random noise. The amplitude of the oscillations is then a random variable, the mean value of which depends on both the internal damping and the external noise. The period of the oscillations is determined largely by interaction within the system although the noise causes a steady loss of phase information. A mechanism of this type is implied in Moran's¹ representation of the Canadian lynx cycle.

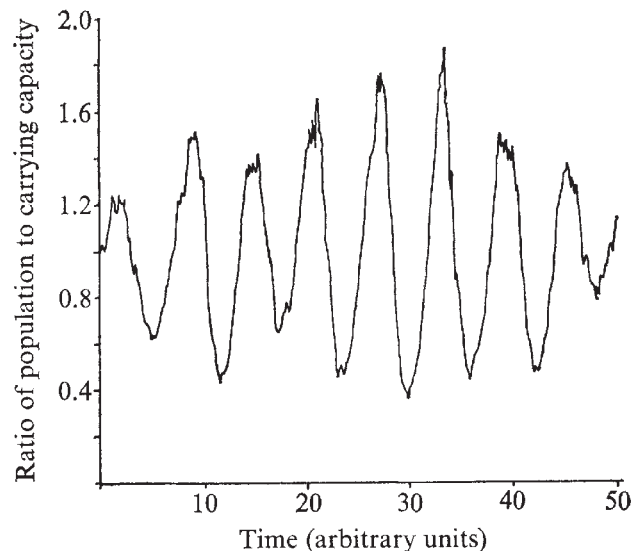


Fig. 1 Quasi-cyclic fluctuations in the time-delayed logistic model. The population is responding to fluctuation in the carrying capacity, K , of the form $K(t) = K_0(1 + \gamma(t))$ where $\gamma(t)$ is Gaussian white noise with standard deviation $\sigma = 0.1$. The intrinsic growth rate r and the time delay τ are chosen so that $r\tau = 1.4$, a value for which the system is underdamped.

- The population is again represented as part of a stable, underdamped system but the control mechanism is assumed to be nonlinear. In a strongly seasonal environment population cycles may then occur as a result of subharmonic resonance. We have previously² illustrated this effect in the time-delayed logistic model with strong periodic variation in the carrying capacity and Bigger (private communication) has suggested that the oscillations³ in populations of the leaf miner *Leucoptera coffeina* (Washb.) may be of this type.

- The population is assumed to be limit cycling (possibly with some environmental fluctuations superimposed on the limit cycle). Both the amplitude and the period of the oscillations are then determined by the interactions within the system. Many examples have been discussed by May⁴.

We have deliberately excluded from the above classification models like Bulmer's⁵ of the Canadian lynx cycle which explain cycles in one population (lynx) as being driven by cycles in another population (snow-shoe hare) but which beg the question of the origin of these driving cycles.

The last two mechanisms—subharmonic resonance and limit cycling—involve complicated nonlinear effects and yet they have received considerably more attention from modellers than the first. Our aim here is to advocate the first mechanism as a paradigm for population cycles, since we feel that its obvious simplicity makes it a strong candidate for representing a seemingly cycling population in a fluctuating environment. Particularly appealing is the fact that the principle of the mechanism follows from a purely linear analysis.

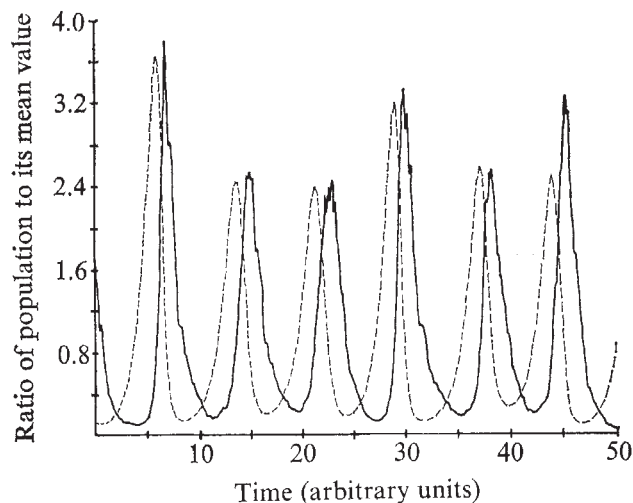


Fig. 2 Quasi-cyclic fluctuations in a damped Lotka-Volterra predator-prey model with a fluctuating predator death rate. The parameters are chosen to make the system underdamped. The continuous and dotted curves denote respectively the predator and prey.

We represent by $x(t)$ the difference between the instantaneous population value and its mean value, and by $\bar{x}(f)$ the Fourier transform of $x(t)$. The square of the modulus of $\bar{x}(f)$ is called the spectral density and is denoted by $S(f)$. If $p(t)$ denotes the fluctuation in some environmental parameter then provided it is small, the nature of the resulting population fluctuations can be deduced from the relationship

$$\bar{x}(f) = T(f)\bar{p}(f)$$

where $T(f)$ is known as the transfer function. We have previously² derived a transfer function for a simple population model and discussed its interpretation in some detail; here we need only note that an underdamped system is characterised by a peak in the transfer function. At high frequencies the transfer function of a stable system is always very small, essentially because a population does not respond to frequencies much greater than the reciprocal of the smallest ecological time constant in the system. Thus if (and only if) Fourier analysis of time series of relevant environmental variables reveals fluctuations which have a flattish spectrum over the range of frequencies for which the magnitude of the transfer function is significant, it is safe for the purpose of population analysis to represent these fluctuations as white noise. Where this idealisation is permissible, the population spectral density is proportional to $|T(f)|^2$.

Whenever environmental fluctuations lead to a peak in the population spectral density, quasi-cyclic behaviour results. Moreover, the argument in the previous paragraph guarantees that any underdamped system will respond in a quasi-cyclic manner to environmental fluctuations with a flattish spectrum. In Fig. 1 we show an example of quasi-cyclic population fluctuations obtained from a time-delayed logistic model with a time delay chosen to correspond to very light damping, and a randomly fluctuating carrying capacity. In Fig. 2 we illustrate the same behaviour in a damped Lotka-Volterra predator-prey model⁸ with random fluctuations in the predator death rate. We have obtained similar cycles when analysing the effects of fluctuating mortality in a single-species model with age structure, and in a model of experiments⁷ in which persistence in a spatially heterogeneous, predator-prey system is achieved through a balance of migration and local extinction. This work will be reported in detail elsewhere.

We have given a simple linear analysis of a mechanism which can generate oscillations. In principle, this treatment ought to be applicable only to very small oscillations, but we have carried out extensive numerical simulations of the above models and conclude that the linear analysis is remarkably accurate in predicting the size and nature of the population fluctuations right up to the point where extinction of the population on an ecological time scale becomes highly probable. Thus we conclude that population cycles of rather large amplitude may occur when an underdamped control system is subject to random environmental noise, and we suggest that this mechanism is worthy of consideration when analysing real population data.

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Monogamy and duetting in an Old World monkey

THE first monogamous mating system among Old World monkeys has been discovered in the Mentawai langur, *Presbytis potenziani*. The only known monogamous anthropoid primates were the Old World gibbons (*Hylobates* spp.)¹ and a few New World monkeys²⁻⁴. Reproductive groups of all known Old World monkeys, represented by approximately 85 species, are either polygamous or promiscuous⁵.

The endemic Mentawai langur is an arboreal folivore confined to Siberut, Sipora, North Pagai and South Pagai islands, 85-145 km west of Sumatra⁶. Our main study population was nine langur groups inhabiting a study area covering 200 ha in central Siberut Island, near the headwaters of the Sirimuri River (1°24'S, 99°1'E). Between July 1972 and November 1974 we spent 447 d and made over 160 h of visual observation of undisturbed Mentawai langurs at this site.

Adult Mentawai langurs exhibit little sexual dimorphism. Ten specimens (6 males, 4 females) show a female-male weight ratio of 0.98 (mean weight: male 6.5 kg; female, 6.4 kg). The only difference in coat coloration between the sexes is the male's conspicuous white genital fur patch.

The 21 groups of langurs we observed were adult pairs with up to four younger animals. Females bear a single infant approximately once every other year. Mean size of nine families studied regularly varied from 3.4 in March, 1973 to 4.0 following the 1974 July-August birth season. Adult pairs remained together for the entire 2.5-y study.

We did encounter six solitary males. These solitary males account for 22% (6/27) of our adult male sample. All adult females observed were monogamously mated. But all-male groups, typical of other polygynous langurs⁴, were never encountered.

Families occupy a home range of about 15-22 ha. Home ranges of adjacent groups overlap extensively, but each contains an exclusively used "core area"⁷ where their sleeping trees (mean = 2 per group) are located. Core areas are situated on major ridge crests, on boundaries between gibbon (*Hylobates klossii*) territories. Intrusion by alien langur males into core

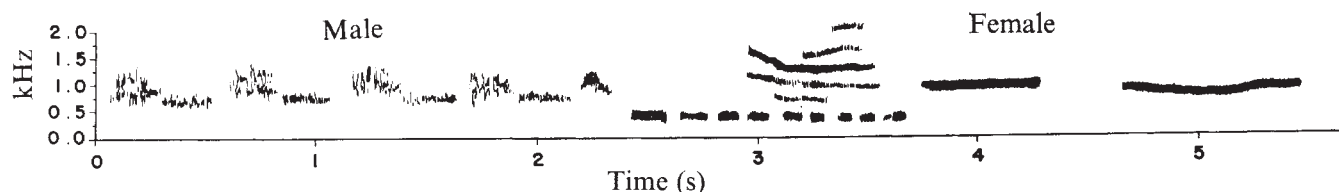


Fig. 1 Ink tracings of audiospectrograms illustrating a typical, duetted intergroup call by a mated pair of Mentawai langurs. The three prolonged (about 0.5 s) sounds terminating the duet are those of the female. The first sound overlaps the staccato terminal trill of the male's vocalisation. Note the two components to the male call; a series of harsh, biphasic sounds corresponding to inhalation and exhalation, followed by a slow, tonal trill. The vocalisations were recorded in the field at 9.5 cm s with a Uher 4400 tape recorder and a Sennheiser 405-S microphone fitted to a parabola 60 cm in diameter. Audiospectrograms were made with a Kay Elemetrics sound spectrograph on wide band setting.

areas results in aggressive inter-male confrontations dominated by loud vocalisations and visual display by owner and trespasser.

Like adult females in other monogamous primates, female Mentawai langurs participate with their mates in vocal and visual displays directed towards adjacent groups. Males emit loud vocalisations (audible to about 1 km), either spontaneously or on hearing or seeing members of other groups. They are comparable to the intergroup "spacing" calls of other male colobines⁶. Male intergroup calls are accompanied by a vigorous jumping display on branches. This shakes the entire tree crown. When the males make intergroup calls, their mates respond by adding a characteristic 3-4-syllable coda (audible to about 0.5 km) to the end of it (Fig. 1). She then leaps from branch to branch, shaking the tree after the male has stopped his display. In other colobines such displays are given only by males⁶.

The vocal portion of the Mentawai langur intergroup display, with its male and female components, resembles the duetting gibbon pairs⁹, titi monkeys², and monogamous tropical birds which remain paired throughout the year^{10,11}. Thus the correlation of duetting with permanent, year-round monogamy

The presence of offspring is significantly correlated ($\chi^2 = 51.9$; $P < 0.001$) with the male's distraction display. For the 35 cases involving either solitary males or mated males with no offspring, langurs fled without giving alarm calls. Occasionally the male coughs harshly (7 of 17 events) if his mate is feeding in another tree. The cough (audible to about 50 m), may alert the female of danger and the male's intention to begin rapid movement. It may precede the louder call (audible to about 1 km) family males emit in response to man (Table 1).

Thus, several interrelated characters closely linked to monogamy in other species typify the Mentawai langur. Its lack of sexual dimorphism extends the correlation between monogamy and monomorphism^{13,14}. The monogamous pair bond is maintained by vocal duetting and mutual visual displays. The distraction display of family males is risky but increases the survival chances of his own offspring.

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Sex chromosome translocations and speciation

SPECIATION is the primary process of cladogenetic evolution. It is generally agreed that genetic divergence sufficient to produce reproductive isolation among populations is

Table 1 Behavioural response of Mentawai langurs to man

	Mated pairs with immature young	Mated pairs only	Solitary males
Distraction display	12	0	0
Cough alert	5	7	0
Rapid flight with no vocalisations	1	10	18
Total (n = 53)	18	17	18

in birds and primates can be extended to the Mentawai langur, and the hypothesis that duetting functions to maintain monogamous pair bonds¹⁰⁻¹³ is supported.

Adult male Mentawai langurs perform distraction displays in response to man, the major and perhaps only predator on primates in Siberut. Other relevant predators such as large raptors or felids do not occur on these islands⁶. Another basis for the relatively great impact of man as a predator on Mentawai primates is the dominance of primates in the island's depauperate mammalian fauna. Primates constitute 82% of all large mammalian prey taken by aboriginal hunters in Siberut. Thus, both the more intensive human predation and the lack of counterselection favouring alternative predator defence strategies probably account for the Mentawai langur's distraction performance in encounters with man.

The adult male distraction display is a loud vocalisation accompanied by branch bouncing, delivered every 20-30 m as he runs through the canopy. Vocalisations resemble the harsh, biphasic syllables of the intergroup call (Fig. 1), but the female remains silent. During this performance the female and immature young often hide silently and motionless in the canopy for periods up to 45 min before leaving. On five different occasions a displaying male made a complete circle after calling 6-8 times, entered the tree his family occupied, then again began a new distraction directed towards the observer.

Table 1 Single-factor genetic basis of $X \cdot Y^L / Y^S$ male mating advantage

(A) Comparisons of yellow and normal bodied males					
	X chromosome	Y chromosome	y^+ mating frequency	n	χ^2 probability
(1)	$X \cdot Y^L, y^+$ and $X \cdot Y^L$	Y^S	0.53	45	0.655
(2)	$X \cdot Y^L$	Y^S, y^+ and Y^S	0.50	260	0.902
(3)	$X \cdot Y^L, y^+ f^+$ and $X \cdot Y^L, f^+$	Y	0.53	45	0.655
(4)	$X, y^+ f^+ car^+$ and $X, f^+ car^+$	Y^S	0.54	117	0.405
(5)	$X, y^+ f^+ car^+$ and $X, f^+ car^+$	Y	0.65	85	0.009*
(6)	X	Y^S, y^+ and Y^S	0.74	39	0.004†
(B) Comparisons of $X \cdot Y^L$ and X males					
	X chromosome	Y chromosome	$X \cdot Y^L$ mating frequency	n	χ^2 probability
(1)	$X \cdot Y^L$ and $X, f^+ car^+$	Y^S	0.57	44	0.366
(2)	$X \cdot Y^L, y^+$ and $X, y^+ f^+ car^+$	Y^S	0.50	62	1.000
(3)	$X \cdot Y^L$ and $X, f^+ car^+$	Y	0.40	50	0.157
(4)	$X \cdot Y^L, y^+$ and $X, y^+ f^+ car^+$	Y	0.56	89	0.244
(C) Comparisons of Y^S and Y males					
	X chromosome	Y chromosome	Y^S mating frequency	n	χ^2 probability
(1)	$X \cdot Y^L$	Y^S and Y	0.63	143	0.003†
(2)	$X \cdot Y^L, y^+$	Y^S and Y	0.69	45	0.017‡
(3)	$X, f^+ car^+$	Y^S and Y	0.64	55	0.059
(4)	$X, y^+ f^+ car^+$	Y^S and Y	0.55	80	0.371

All mating chambers were run with 12 males of each genotype and 24 $X \cdot Y^L / X \cdot Y^L, y f car$ females. Unless otherwise indicated males were also $y f car$. Where males were not phenotypically distinguishable, the tips of the wings were clipped to allow identification; different genotypes were clipped in replicate chambers. No significant effect of clipping was observed. n, Total number of matings observed. Goodness-of-fit χ^2 probabilities were corrected for continuity when the associated probability was greater than 0.10.

* $P < 0.01$.

† $P < 0.005$.

‡ $P < 0.05$.

acquired in the allopatric state¹⁻⁴. One of the major problems of evolutionary genetics is, then, characterisation of the genetic differences which actually produce the reproductive and sexual isolation characteristic of species. A partial answer to this problem has been provided by estimating the degree of structural gene divergence over various taxonomic levels—populations, subspecies, semispecies, sibling species and morphologically distinguishable species^{5,6}. The general picture emerging from these studies is moderate structural gene differentiation at the subspecies level. Once reproductive isolation has been achieved, little additional change seems to be required to establish sexual isolation between subspecies. There are, however, exceptions to this generalisation^{7,9}, and they do not seem to be infrequent. Speciation may occur as a result of chromosomal rearrangements, accompanied by little, if any, detectable change in the structural genes usually studied.

Attempting to explain the preponderance of sterility and inviability in the heterogametic progeny of hybrid matings, Haldane¹¹ suggested that sex chromosome translocations might be the cause. Tracey¹² estimated fixation probabilities

of an $X \cdot Y^L$ and a Y^S chromosome in laboratory populations of *Drosophila melanogaster*. The estimated probability of fixation exceeded neutral expectation for both of these translocation chromosomes; moreover $X \cdot Y^L / Y^S$ males had a mating advantage compared with $X \cdot Y^L / Y$ males when the females competed for were $X \cdot Y^L / X \cdot Y^L$. We have examined the genetic basis of this mating advantage and found that it is primarily determined by the Y^S chromosome, with the Y^L segment attached to the X and the yellow allele playing significant but lesser roles. In addition we have found that a population of *D. melanogaster* bearing $X \cdot Y^L, Y^S$ and the yellow allele exhibits significant sexual isolation with respect to a karyotypically normal strain homozygous for the eye mutant *sparkling-poliert*.

The $X \cdot Y^L$ chromosomes of all females used to test mating advantage carry the markers *yellow*, *forked* and *carnation*. Three Y chromosomes have been used: a normal Y, a Y^S which lacks the Y^L arm but is fertile when $X \cdot Y^L / Y^S$ and a Y^S bearing a translocated normal allele of *yellow* designated Y^S, y^+ . Observation chambers¹³ were used to study mating; the genotypes used were produced by crossing to

Table 2 Multifactor genetic basis of $X \cdot Y^L / Y^S$ male mating advantage

	X chromosomes	Y chromosomes	A mating frequency	n	χ^2 probability
	(A)	(B)	(A)	(B)	
(1)	$X \cdot Y^L$	compared with X	Y^S	Y^S, y^+	0.71
(2)	$X \cdot Y^L$	compared with $X \cdot Y^L$	Y^S, y^+	compared with Y	0.71
(3)	$X \cdot Y^L, y^+$	compared with $X \cdot Y^L$	Y^S	compared with Y	0.67
(4)	X	compared with X	Y^S, y^+	compared with Y	0.76
(5)	X	compared with $X \cdot Y^L$	Y^S	compared with Y^S, y^+	0.31
(6)	X	compared with $X \cdot Y^L$	Y	compared with Y^S, y^+	0.09
(7)	$X, y^+ f^+ car^+$	compared with $X, f^+ car^+$	Y	compared with Y^S	0.93

Male genotypes were combinations of columns A/ /A and B/ /B; as in Table 1, female genotypes were always $X \cdot Y^L / X \cdot Y^L, y f car$, and 24 females were used in each chamber. Twelve males of each genotype were used and they were $y f car$ except where wild alleles are noted. n, Total number of matings observed.

Goodness-of-fit χ^2 probabilities were corrected for continuity where the associated probability was less than 0.10.

* $P < 0.005$.

† $P < 0.05$.

a locally collected wild-type stock and selecting the desired recombinants.

Yellow males are known to mate less frequently than their normal counterparts^{14,15}; therefore, we first compared yellow and normal bodied males (Table 1). There is no demonstrable difference in mating success between these males when they are fertile except for flies with normal chromosomes. X/Y males of normal body colour show a significant mating advantage. Similarly, the presence or absence of the Y^L segment on the X chromosome does not significantly affect mating success when this is the major genetic difference between competing males. Note, however, that the equivalence of yellow and normal male mating frequency does not hold when the males lack the Y^L segment and the Y^S carries a y⁺ marker. When Y^S- and Y-bearing males are compared, the Y^S males do significantly better when they are fertile regardless of body colour. The absence of the Y^L segment on the X not only makes X/Y^S males sterile, but also eliminates the mating advantage for flies of normal colouration. When yellow X/Y^S and X/Y males competed, a nearly significant excess of X/Y^S matings was observed. If this difference is real, it seems that the yellow allele and the Y^L segment interact with the Y^S to produce the mating advantage observed in X·Y^L/Y^S males.

Further evidence of interaction is presented in Table 2 which summarises mating chamber studies in which both the X and Y chromosomes of males differed. Comparison of studies 1 and 5 (Table 2), in which all Y chromosomes were Y^S but the yellow marker and the X·Y^L chromosome were juggled, suggests that yellow males are more readily accepted by X·Y^L/X·Y^L females when the males carry the X·Y^L. In the presence of a normal X chromosome, the yellow mating advantage disappears. The comparisons of X·Y^L and X males (Table 1) indicate that this difference is attributable to a yellow by X·Y^L interaction. Males of normal body colour mate more frequently when they are of normal karyotype or carry a Y^S marked with y⁺ (Table 1, A5 and 6; Table 2, studies 2, 4, 5 and 6). The body colour equivalency apparent in Table 1, however, indicates that this advantage is an effect of the Y^S rather than the y⁺ allele. Moreover, X/Y^S males are sterile; any mating advantage of such males is meaningless in an evolutionary context.

Thus, mating advantage of X·Y^L/Y^S males is primarily affected by the Y^S chromosome and augmented by a yellow-X·Y^L interaction. As X·Y^L/Y^S is fitter than X/Y when females are X·Y^L/X·Y^L, it is important to assess the degree to which an X·Y^L-Y^S system is sexually isolated from normal, X-Y populations of *D. melanogaster*. Conclusive tests of the degree of sexual isolation attributable to the translocation and to the yellow marker will require extensive homogenisation of genetic background. We have, however, obtained exciting results in a preliminary test. Males and females from the X·Y^L, y f car; Y^S stock were placed in observation chambers with a wild strain containing the marker gene sparkling-poliert eye. The degree of homogametic mating exceeds 82%; 70 of 85 matings were between males and females of the same stock. This difference is highly significant and yields a sexual isolation coefficient equal to 0.65±0.08; a value in excess of those obtained for populations and subspecies of *D. willistoni*¹⁶, but within range of values obtained for semispecies of *D. paulistorum*¹⁷.

To the extent that we may extrapolate from laboratory studies, these and previous¹² results suggest that speciation may occur with minimal genic change. The fixation of X·Y^L and Y^S in an isolated population is a virtual certainty given sufficient time. In the absence of other change this X·Y^L-Y^S population is reproductively isolated from its progenitor. The additional substitution of the yellow allele, which is itself advantageous in some conditions¹⁸, engenders both a greater mating advantage and a rather high degree

of sexual isolation. In the light of the high frequency of sex chromosome polymorphisms, within and among species^{19,20}, and the low degree of genetic divergence between some of these species^{7,9}, Haldane's explanation of reproductive isolation requires serious consideration.

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Lipoprotein of Gram-negative bacteria is essential for growth and division

THE lipoprotein of Gram-negative bacteria has become a cell surface component of central interest following its characterisation by Braun and coworkers¹. This small protein (molecular weight 7,500) is unique in containing as its N-terminal amino acid a glycylcysteine to which three fatty acids are covalently bound². It is also unusual in its subcellular distribution: one-third of the total lipoprotein is covalently linked to the cell wall peptidoglycan while the remaining two-thirds exists in an unlinked or "free" form in the outer membrane⁴. Free and murein-linked lipoprotein seem to be identical in structure^{5,6}, and pulse-chase experiments indicate that free lipoprotein is the precursor to murein-linked lipoprotein⁴. Braun's lipoprotein or an immunologically related protein has been found in a wide variety of Gram-negative organisms⁷⁻⁹, and in *Escherichia coli* is present in about 3 × 10⁵ copies per cell¹. These observations suggest that lipoprotein performs some essential function(s); however, the nature of that function is unknown. It has been suggested that lipoprotein may have a role in anchoring the outer membrane to the cell wall³, or function in transporting small molecules through the outer membrane⁸ or be involved in cell division^{10,11}. To clarify the role of Braun's lipoprotein, we have isolated a mutant deficient in its synthesis.

The procedure used for isolation of the mutant was based on some of the unusual properties of the lipoprotein. The primary amino acid sequence of Braun's lipoprotein has been determined¹²; it has been found to lack five amino acids, including proline, histidine and tryptophan. Hirashima and Inouye¹³ have demonstrated that during starvation for these amino acids, *E. coli* continues to synthesise lipoprotein, although synthesis of other proteins is drastically reduced. This observation suggested the possibility of enriching for a lipoprotein mutant by starving cells for proline, histidine and tryptophan, labelling with arginine, and performing a suicide selection. Since 42% of the total radioactivity incorporated by our strain

after amino acid starvation was found in lipoprotein (as compared to 3% in normal conditions), on prolonged storage a mutant which had not synthesised lipoprotein should survive longer than wild type. Two additional considerations taken into account were that lipoprotein might be essential for growth, and that the suicide would only enrich for mutants which either did not synthesise the protein or degraded it very rapidly. This prompted the use of a strain carrying a temperature-sensitive amber suppressor, which would allow the isolation of a conditional amber mutant in lipoprotein.

To perform the suicide selection, a strain which carried a temperature-sensitive suppressor and which could be starved for the appropriate amino acids was constructed by transducing D117 (an *E. coli* K12 strain of genotype *pro⁻his⁻trp⁻Su⁻Δlac⁻F⁻lac⁻* amber) with bacteriophage P1 grown on *E. coli* MB188 (*trp⁻am SuIII-A81 ts* from K. Begg). A transductant was selected which was phenotypically *lac⁺trp⁺* at 30 °C and *lac⁻trp⁻* at 42 °C; the transductant also showed temperature sensitivity in its ability to allow growth of amber phage. It was cured of the episome by acridine orange. The resulting strain

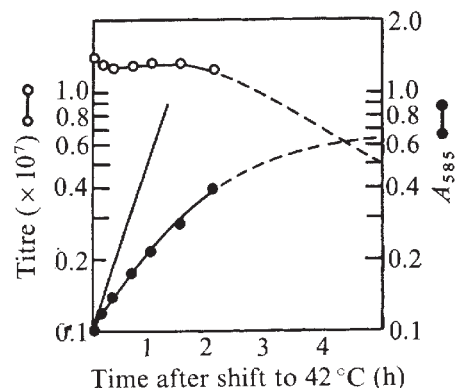


Fig. 2 Effect of temperature shift on growth and viability of ST715. ST715 was grown at 30 °C in L broth²⁶ and at time 0 diluted 1/10 into prewarmed (42 °C) L broth. Viable counts (O) and absorbance (●) were measured. The solid line is a growth curve (absorbance) of the parent (M7) at 42 °C.

M7 (*pro⁻his⁻trp⁻am SuIII-A81 ts*), was mutagenised to 5% survival with nitrosoguanidine¹⁴ diluted 1/10, and allowed to grow overnight at 30 °C in E medium¹⁵ supplemented with the required amino acids. The culture was filtered, washed, resuspended in 50 ml E medium lacking proline, histidine, and tryptophan, and incubated at 42 °C for 60 min. ³H-arginine (50 μCi ml⁻¹, specific activity 28.7 Ci nmol⁻¹) was added, and incubation at 42 °C continued for 90 min. The cells were collected by filtration, washed with minimal salts containing unlabelled arginine (500 μg ml⁻¹), and then washed with cold water to remove pools of unincorporated radioactive arginine. The cells were resuspended in cold E salts and stored at 4 °C. Viability was measured at intervals by plating at 30 °C. The cell titre decreased exponentially, and had fallen from an initial value of 1×10^8 ml⁻¹ to 3×10^3 ml⁻¹ after 45 d. Unlabelled control cells decreased in viability by less than 50% during this time. After 45 d, all surviving cells were plated on minimal plates and screened for temperature-sensitive growth on nutrient agar plates. Of 23,000 total survivors obtained from several experiments, 513 were temperature sensitive, and these were assayed for lipoprotein synthesis at 30 and 42 °C.

One of the temperature-sensitive survivors was found to synthesise reduced amounts of murein-linked lipoprotein at 42 °C (Table 1). Murein-linked lipoprotein accounts for 3.2% of the arginine incorporated into the envelope fraction of M7 at 30 °C; this figure is slightly higher at 42 °C. In contrast, strain ST715, although showing normal lipoprotein levels at

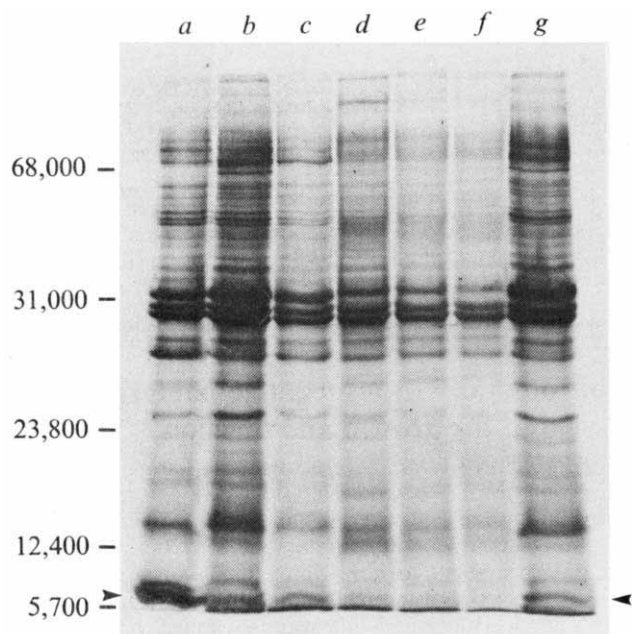


Fig. 1 SDS-acrylamide gel electrophoresis of envelope fractions from M7 and ST715 grown at 30 and 42 °C. M7 and ST715 were grown at 30 °C in minimal medium, filtered, and resuspended in medium containing cold arginine (2 μg ml⁻¹). A portion of each culture was shifted to 42 °C, and aliquots withdrawn after 30, 60, and 90 min. Each aliquot was labelled at 42 °C for 30 min by the addition of ¹⁴C-arginine (1 μCi ml⁻¹, specific activity 277 mCi mmol⁻¹). Control cultures (M7 and ST715 at 30 °C, M7 at 42 °C) were labelled 30 min after shift. Cultures were collected and envelope fractions prepared. These were dialysed overnight against 0.01 M Tris-HCl pH 6.8 at 4 °C, and collected by centrifugation at 90,000g for 60 min. Envelope fractions were solubilised in sample buffer²³ containing 0.5% SDS and applied to a 12.5% acrylamide slab gel²⁴ containing 0.5% SDS along with molecular weight markers. The gel was stained with Coomassie brilliant blue²⁵, dried and autoradiographed. Sample wells contain: a, M7 labelled at 30 °C, 60,000 c.p.m. applied; b, M7 labelled at 42 °C, 115,000 c.p.m. applied; c, ST715 at 30 °C, 40,000 c.p.m. applied; d, ST715 after 30 min. at 42 °C, 50,000 c.p.m. applied; e, ST715 after 60 min at 42 °C, 30,000 c.p.m. applied; f, ST715 after 90 min at 42 °C, 27,000 c.p.m. applied; g, same as b. Molecular weight standards were bovine serum albumin (68,000), DNase (31,000), trypsin (23,800), cytochrome c (12,400) and insulin (5,700). Free lipoprotein migrates immediately behind the front and is indicated by an arrow. The band of slightly higher molecular weight migrating behind free lipoprotein is presumably murein-linked lipoprotein which has been released by endogenous murein hydrolases. This assumption is based on the fact that neither band contains histidine (data not shown), and that the synthesis of both seems to decrease in a coordinate fashion during incubation of ST715 at 42 °C.

Fig. 3 Effect of temperature shift on morphology of ST715. ST715 was grown in L broth at 30 °C (a), and shifted to 42 °C for 3 h (b). Cells were collected by centrifugation, resuspended in 0.01 M Tris pH 6.8, and stained with 2% uranyl acetate. Micrographs were taken in a Joelco 100B electron microscope at a magnification of $\times 5,000$.



Table 1 Assay of murein-linked lipoprotein in parent and mutants grown at 30 and 42 °C

		Total envelope (¹⁴ C-Arg c.p.m.)	Lipoprotein (c.p.m.)	Lipoprotein/total envelope ratio (%)
M7	30 °C	73,968	2,392	3.2
	42 °C	77,536	3,304	4.3
ST715	30 °C	39,276	1,255	3.2
	42 °C	80,055	636	0.8

This assay is based on the observation²⁰ that murein-linked lipoprotein can migrate on chromatograms in an isobutyric acid-ammonia solvent after it is released from murein by lysozyme digestion. ST715 and M7 were grown at 30 °C in E medium supplemented with required amino acids and arginine. In the early phase of exponential growth, the culture was filtered and cells resuspended at 30 °C in medium containing arginine (4 µg ml⁻¹). After 10 min, a portion of each culture was shifted to 42 °C and incubated for 30 min; control cultures remained at 30 °C. ¹⁴C-arginine (specific activity 277 mCi mmol⁻¹) was added to 0.5 µCi ml⁻¹, and incubation continued for 30 min. Cultures were chilled, collected by centrifugation at 4 °C, washed once with cold saline and re-suspended in 1 ml cold 0.01 M Tris HCl pH 6.8. The cells were ruptured by sonication, centrifuged for 5 min at 3,000g to remove unbroken cells, and then centrifuged at 90,000g for 60 min to collect envelopes. These were resuspended in a small volume of water and boiled for 5 min. A small aliquot was spotted on Whatman 3MM chromatography paper for direct counting; the remaining sample was applied in two equal portions to Whatman 3MM paper and chromatographed overnight in isobutyric acid-1 N NH₄OH (5:3) (descending chromatography). This procedure separates murein-linked lipoprotein (origin) from free lipoprotein and other envelope proteins which migrate with an *R_f* of 0.8. Chromatograms were dried, and a 2-cm region around the origin cut out. One of each duplicate sample was incubated overnight at 37 °C in 2 ml 0.5 N ammonium acetate containing egg white lysozyme 500 µg ml⁻¹; the control was incubated in buffer alone. Incubation with lysozyme releases the lipoprotein from the cell wall material at the origin but does not liberate the lipoprotein from the paper.

The origins were washed twice with water, dried, sewn on to Whatman 3MM paper, and rechromatographed in isobutyric acid-1N NH₄OH (5:3) for 4 h. Murein-linked lipoprotein migrates with an *R_f* of 0.8 during this chromatography. Chromatograms were dried, cut into 2-cm pieces and counted. Each value was corrected for counts released during the control incubation (from 10–20% of the value obtained using lysozyme), and samples were normalised by comparing each lipoprotein value to total envelope counts applied. This assay is specific for murein-linked lipoprotein since there is little release of radioactivity in the absence of lysozyme; the released material contains palmitic acid and diaminopimelic acid but not histidine; when eluted from the chromatogram and subjected to SDS-acrylamide gel electrophoresis, all the material comigrates with *in vivo*-labelled murein-linked lipoprotein (data not shown).

30 °C, shows a fourfold decrease in incorporation into lipoprotein after 60 min at 42 °C. A second survivor also showed a twofold decrease in murein-linked lipoprotein at 42 °C as well as slightly reduced levels at 30 °C (data not shown); this strain was very unstable and further studies were performed on ST715 only.

To determine whether ST715 also showed a temperature-dependent reduction in synthesis of free lipoprotein, M7 and

ST715 were incubated at 30 and 42 °C for various periods of time and then labelled with ¹⁴C-arginine. Whole envelope fractions were prepared and subjected to sodium dodecyl sulphate (SDS)-acrylamide gel electrophoresis as shown in Fig. 1. This gel does demonstrate a decrease in synthesis of lipoprotein in ST715 as a function of time of incubation at 42 °C; this decrease is quite gradual, and some synthesis is still detectable after 2 h at 42 °C, presumably reflecting leakiness of the temperature-sensitive suppressor. Other changes in the envelope fraction of ST715 at 42 °C can be seen; notably the appearance and gradual disappearance of a protein band of high molecular weight (~90,000) and the disappearance of a band of ~70,000. The relationship of these changes to the lipoprotein lesion is unclear and will be studied in revertants.

The effects of lipoprotein deficiency on cell physiology were studied by shifting an exponentially growing broth culture of ST715 from 30 to 42 °C (Fig. 2). This shift results in an abrupt cessation in the increase of viable counts. Cell mass continues to increase, however, and filaments are formed (Fig. 3). After several hours at 42 °C, viability begins to decrease slowly; at this time cells are 4–8 times their normal length. Thus, continued lipoprotein synthesis seems necessary for cell division.

To determine whether lipoprotein deficiency, filament formation and temperature-sensitive growth were the result of a single mutation, revertants which could grow at the non-permissive temperature were selected. These were found to occur at a frequency of approximately 10⁻⁹. Ten revertants were selected for further study, and all were found to have increased levels of murein-linked lipoprotein at 42 °C. Two revertants, E4 and E10, which showed normal growth rates at 42 °C, were analysed for murein-linked lipoprotein and found to have levels comparable to M7 (Table 2). Interestingly, E10 seems to be a wild-type revertant, whereas E4 has apparently reverted to temperature-independent suppression. The fact that activation of a suppressor at 42 °C enables E4 to grow and make lipoprotein at this temperature suggests that ST715 carries an amber mutation. Other revertants studied seemed to be partial revertants, which grew with a greater than normal generation time at 42 °C, and, showed only partial restoration of bound lipoprotein levels at 42 °C (ranging from a two- to fourfold increase over mutant levels in an experiment in which the parental level was 7 times that of ST715). This may indicate that lower amounts of lipoprotein than observed in the parent are sufficient to support growth. The results shown in Table 2 also demonstrate that growth at 42 °C, filament formation, and lipoprotein content are properties linked by transduction. These results indicate that all properties of the mutant are due to a single mutation, and suggest that lipoprotein may be necessary for normal growth and division.

Wu and Lin²¹, using a suicide technique similar to that described here, have recently isolated a mutant deficient in linking lipoprotein to murein. Their mutant, like ST715, shows temperature sensitivity in growth and division. Since the strain

Table 2 Properties of revertants and transductants of ST715

Strain	Growth at 42 °C		Plates T4amB25		Morphology		Murein-linked lipoprotein (at 42 °C)/ total envelope ratio
	+tryp.	-tryp.	30 °C	42 °C	30 °C	42 °C	
M7	+	—	+	—	rod	rod	4.5%
ST715	—	—	+	nd	rod	fil	0.5%
E4	+	+	+	+	rod	rod	4.0%
E10	+	—	+	—	rod	rod	3.1%
TD4	+	—	+	—	rod	rod	5.3%
TD6	+	—	+	—	rod	rod	3.6%

Revertants of ST715 were isolated by spreading approximately 3 × 10⁹ cells on minimal plates and incubating at 39 °C. Revertant clones were purified, tested for amino acid requirements and streptomycin resistance, and grown in minimal medium at 42 °C to determine growth rate. Murein-linked lipoprotein was measured on cultures which had been pregrown at 30 °C, shifted to 42 °C for 60 min, and then labelled at 42 °C for 30 min with ¹⁴C-arginine. Growth requirement for tryptophan was determined on plates; phage sensitivity was measured by plating efficiency at 30 and 42 °C; and morphology examined by phase microscopy.

P1 transduction was performed as described²⁷ using *E. coli* K12 strain GR2131 (genotype *pro-leu-trp-gal-man-xyl-mal-lac-phoA purE* from G. Willsky) as a donor. Transductants which were able to grow at 42 °C were isolated, purified, and tested for amino acid requirements. Two transductants, TD4 and TD6, were assayed for murein-linked lipoprotein and tested for other properties as described above.

isolated by Wu and Lin seems, however, markedly deficient in linking lipoprotein to murein at the permissive temperature, it is possible that murein-linked lipoprotein does not have an essential role in growth and division of *E. coli*. This would imply that it is the deficiency in free lipoprotein which is responsible for the phenotype of ST715. In addition, until it can be shown that ST715 carries a lesion in the structural gene for lipoprotein, it remains possible that ST715 is affected in growth and lipoprotein through a mutation which affects both properties indirectly. An example would be a protease responsible for processing lipoprotein and other essential proteins.

Mutants with reduced levels of other major outer membrane proteins (of molecular weight 30,000–40,000) have been described^{14,15} as well as mutants which lack one^{16,17} or all¹⁸ of these proteins; however, such mutants are viable and morphologically normal. This is in contrast to ST715, which is unable to grow and divide at the non-permissive temperature when lipoprotein formation is greatly reduced. Braun's lipoprotein thus may serve a more vital function(s) than do these other major outer membrane proteins. Further studies with the lipoprotein mutant described here should define the role of this major component of the outer membrane in cell growth and division.

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Prevention of the contagious spread of feline leukaemia virus and the development of leukaemia in pet cats

CLUSTERING of cases of feline lymphosarcoma (LSA) or leukaemia has been observed by veterinarians for many years^{1–4}. The first anti-feline leukaemia virus (FeLV) serum was reported in 1969⁵ and by 1970 a simple indirect immunofluorescent antibody (IFA) test for the detection of FeLV in the peripheral blood of infected cats had been developed⁶. Using this test we found that 33% of the healthy pet cats exposed to cats with FeLV-associated diseases were infected with FeLV. All the cats known not to have been exposed to cats with FeLV-associated diseases were uninfected and only 0.31% of the stray cats, with an unknown history of FeLV exposure, were infected³. Once persistently viraemic with FeLV, most

cats remain infected for their entire lives. These results showed conclusively that FeLV is transmitted between cats by infection or contagion (that is, horizontally), in contrast to the oncornaviruses of inbred mice which are predominantly transmitted genetically from the parents to the offspring by means of the chromosomes (that is, vertically)⁷.

FeLV causes four fatal diseases in pet cats. The diseases are: (1) LSA^{4,5}, (2) non-regenerative anaemia^{8,9}, (3) a panleukopenia-like syndrome³, and (4) thymic atrophy¹⁰. FeLV is also associated with, but not yet proven to be the cause of, various myeloproliferative disorders¹¹ and foetal abortions and resorptions (F. Goldsmith and W. D. H., Jr, unpublished)^{12,13}. Healthy FeLV-infected cats have a greatly increased chance of developing one of the FeLV-related diseases or of developing secondary diseases due to the immunosuppressive effects of FeLV^{12,14}.

The spread of an infectious disease agent may be prevented by various methods. These are: (1) vaccination, (2) killing the infectious agent in the environment by disinfection, and (3) removal of either the infected sick animal, the healthy carrier host or the vector(s). In veterinary medicine, when vaccination is not available, removal of the carrier host is commonly used to prevent the spread of infectious disease agents¹⁵.

Our earlier epidemiological studies showed that FeLV is an infectious agent for pet cats³. The ultimate goal of any epidemiological study is to prevent the spread of a disease by identifying the aetiological agent so that it can be removed from the environment. We report here the successful use of an FeLV test and removal programme to prevent the spread of FeLV, and the diseases it causes, among pet cats. The FeLV test and removal programme consists of the identification and removal, by euthanasia or isolation, of FeLV-infected healthy cats from contact with uninfected cats¹⁶. The IFA test for FeLV detects FeLV gs antigens in the peripheral blood leukocytes and platelets of infected cats^{3,6} and we have previously reported that a positive IFA test result means that the cat is viraemic^{6,13}.

In most multiple cat households in this study an index FeLV test was performed on a sick or healthy cat to establish the FeLV status of the households. The index test-positive cat was removed and all the remaining healthy cats in the household were then tested for FeLV (initial test). In the programme, any exposed healthy cats which were found to be infected with FeLV were removed immediately. After the initial test of all the healthy cats, the household was quarantined so that no cats were allowed to leave and no new cats were brought into the household. After a 3-month interval a second test for FeLV was performed on all the remaining healthy cats in the household and any secondarily infected cats were removed. Cats which were FeLV uninfected in the initial test but which were found to be FeLV infected in the second test will be referred to as secondarily infected cats in this paper. Two IFA tests for FeLV are required for exposed cats because the natural incubation period for the establishment of viraemia can be as long as 3 months, and there is a possibility that an FeLV infection, occurring just before the first test, would not be detected in that test¹³. If any healthy cats were positive in the second IFA test they were removed from the household and a third test was conducted 3 months later. When all the cats remaining in a household after the infected cats had been removed were negative in two consecutive IFA tests, conducted 3 months apart, the household was considered FeLV free. All new cats which were introduced into an FeLV-free household were tested for FeLV before they were allowed to have contact with the uninfected cats.

A total of 1,260 healthy cats, from 65 households in which cats had developed FeLV-related diseases and from 11 breeding catteries, have been studied (Table 1). In 51 of these households the test and removal programme was implemented, while in 25 households the owners chose not to implement the programme. These 25 households served as controls and were therefore compared with the 51 households in which the

Table 1 Disease and FeLV status of household groups before the FeLV test and removal programme

Household groups	Number of households	Total cats	Number of cats with FeLV diseases			Index FeLV test*		Total cats remaining after diseased cats died
			Lymphosarcoma	FeLV anaemia	Other	Household status FeLV infected	Not tested	
Households where FeLV-infected healthy cats were removed	51	923	42	6	28	37	14†	847
Households where FeLV-infected healthy cats were not removed	25	472	37	4	18	23	2‡	413
								Total 1,260

*An index FeLV test was the first IFA test of a cat in a household.

†Index FeLV tests were not performed in 10 breeding catteries because no cats had previously developed an FeLV disease. No index tests were performed in the other 4 households where cats had developed FeLV diseases because these cats had died before our study began.

‡An index FeLV test was not performed in 1 household (a breeding cattery) because no cat had previously developed an FeLV disease. An index test was not performed in the other household where a cat had developed an FeLV disease because this cat had died before our study began.

Table 2 Summary of the test and removal programme to prevent the contagious spread of FeLV in healthy pet cats

Household groups	Number of households	Number of healthy cats in households	Number of healthy cats tested	Initial test of healthy cats FeLV results		Initial infection rate	Number of healthy cats removed	Second test* of healthy cats FeLV results		Number of healthy cats which became secondarily† infected	Secondary infection rate of healthy cats
				Infected	Uninfected			Infected	Uninfected		
Households where FeLV-infected healthy cats were removed	51	847	847	190	657	22.4%	190	3	654	3	0.46%
Households where FeLV-infected healthy cats were not removed	25	413	413	129	284	31.2%	0	184	229	55	19.3%

*The second FeLV test was performed at least 3 months after the initial FeLV test.

†A secondarily infected cat is one which was FeLV uninfected in the initial test but was found to be infected in the second test.

Table 3 Summary of disease development in household groups

Table 3. Summary of disease development in secondarily FeLV-infected healthy cats											
Household groups	Number of households	Number of initial test FeLV-uninfected cats	Number of healthy cats which became secondarily infected	Lymphosarcoma	Other FeLV† caused diseases	Other non-FeLV‡ caused diseases	Euthanised	Alive	Number of cats which died naturally	Natural mortality rate in first test FeLV-uninfected§ healthy cats	Natural mortality rate in secondarily FeLV-infected healthy cats
Households where FeLV-infected healthy cats were removed	51	657	3	—	—	—	3	—	—	—	—
Households where FeLV-infected healthy cats were not removed	25	284	55	7	6	11	9	22	24	8.5%	52%

*A secondarily infected cat is one which was FeLV uninfected in the initial test but was found to be infected in the second test.

†4 cats with FeLV non-regenerative anaemias; 2 cats with the panleukopenia-like syndrome.

‡3 cats with feline infectious peritonitis; 8 other diseases—these diseases occur frequently in FeLV-infected cats due to the immunosuppressive effects of the virus.

§These cats subsequently became secondarily infected with FeLV.

owners implemented the FeLV test and removal programme. A total of 847 cats were tested for FeLV in the 51 households in which the programme was implemented (Table 2). Of these 847 cats, 190 cats were found to be infected in the initial FeLV test (an initial infection rate of 22.4%) and were immediately removed from contact with the remaining 657 uninfected cats. Only 3 of these 657 uninfected healthy cats were found to be infected in the second test. In the 3-month period between the two IFA tests, 99.54% of the initially uninfected cats therefore remained uninfected (a secondary infection rate of 0.46%).

In the 25 households where the FeLV-infected healthy cats were not removed, and thus remained in continual contact with other cats in the households, a total of 413 cats were tested for FeLV and 129 were found to be infected in the initial test (an initial infection rate of 31.2%). Of the remaining 284 uninfected healthy cats in these households, 55 were found to be infected in the second test (a secondary infection rate of 19.3%) (Table 2). Thus only 80.7% of the initially uninfected cats remained uninfected in the 3-month period between the two tests. The secondary FeLV infection rate in the households in which the FeLV test and removal programme was not implemented was 42 times greater than that in households in which the programme was implemented. This difference is highly significant ($P \leq 0.0001$) by the χ^2 test. Thus the natural contagious spread of FeLV between cats was effectively prevented by the FeLV test and removal programme.

We have reported previously that FeLV-infected cats have a significantly higher chance of developing one of the FeLV-related diseases³ and a much higher mortality rate than uninfected cats¹⁷. There was a marked difference in the occurrence of the disease and the mortality rate of cats living in the two household groups. The development of FeLV-related diseases was prevented in healthy cats living in the 51 households in which the test and removal programme was implemented. In contrast, in the 25 households where the programme was not implemented, 7 cats have developed LSA, 4 cats have developed FeLV non-regenerative anaemia and 13 cats have developed other FeLV-associated diseases within the 2-yr observation period (Table 3).

The natural mortality rate for the 3 healthy initially uninfected cats which became secondarily infected in those households in which the programme was implemented was not determined since the cats were euthanised immediately after they were found to be infected and thus did not have a chance to develop disease. In the 25 households in which the programme was not implemented 24 of the 55 secondarily infected cats subsequently died resulting in a mortality rate of 52%. These 24 deaths represent a natural mortality rate of 8.5% of the 284 initially FeLV-uninfected healthy cats (Table 3).

Some healthy cats living in households where the control programme was not implemented remained uninfected even though they were continually exposed to viraemic cats. To ensure that the test and removal programme, rather than FeLV neutralising antibody, was responsible for the protection of uninfected cats, cats living in selected households which implemented the programme were examined for neutralising antibody. Forty-one per cent of these cats tested had protective ($\geq 1:10$) neutralising antibody titres. Fifty-nine per cent of the cats thus had non-protective antibody titres and were susceptible to FeLV infection. Because of the implementation of the test and removal programme, however, these susceptible cats were not continuously exposed to FeLV and thus remained uninfected and did not develop any FeLV related diseases.

Pet cats of all ages are susceptible to FeLV infection in their natural household environments. The median age when FeLV was first detected in 49 of the secondarily infected pet cats in our study was 24.0 months. The median age at which these cats were last tested and found to be uninfected was 18.0 months. Therefore, between 18 and 24 months these adult pet cats became infected with FeLV in their household environments.

The FeLV test and removal programme is currently being used by many veterinarians to protect FeLV-uninfected cats

from FeLV infection and disease development¹⁶. The diseases caused by, or associated with, FeLV can be prevented by the test and removal programme. This is significant since diseases caused by and associated with FeLV are probably the most common killer of pet cats.

The results obtained by identifying and removing the healthy inapparent FeLV-infected carrier cats show for the first time that it is possible to prevent the contagious spread of a mammalian oncornavirus in the natural environment. FeLV and the diseases it causes may ultimately be controlled by a combination of the test and removal programme and vaccination.

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Strain differences in the autoimmune response of mice to acetylcholine receptors

EXPERIMENTAL autoimmune myasthenia gravis (EAMG) has been induced in rabbits¹⁻⁴ guinea pigs^{5,6} rats^{4,6} and monkeys⁷ by injection of the acetylcholine receptor (AChR) isolated from electric fish. The experimental disease, together with the demonstration of humoral⁸⁻¹⁰ and cellular¹¹ immune responses to AChR in patients with myasthenia gravis (MG), verifies hypotheses^{12,13} about the immunopharmacological block of AChR in MG. An experimental model disease in mice is needed for studies of the genetic aspects of MG and the role of the thymus as a specific antigen target, and as a source for immunocompetent helper and suppressor cells. We describe here the induction of EAMG in several inbred strains of mice and demonstrate different susceptibility to the disease in strains representing different haplotypes of the major histocompatibility complex (H-2). We describe some humoral and cellular aspects of the disease and demonstrate that in a susceptible mouse strain both humoral and cellular immune response to self-AChR are elicited. Finally we show that AChR behaves as a thymus-dependent antigen.

Mice were injected twice with a 9-week interval with 10 µg of purified *Torpedo californica* AChR (specific activity 10 nmol of toxin per mg protein) per mouse. The

immunogen was emulsified in complete Freund's adjuvant (CFA) and injections were made into the footpads. Ten days or more after the second injection clinical signs of EAMG were observed in mice of the following strains: A/J and B10.A with the H-2^a haplotype, C57BL/6, C3H.SW and CWB with H-2^b, BALB/c and DBA/2 with H-2^d, AKR/Cu and CKB with H-2^k. The disease was not found in any mice with H-2^a and H-2^s haplotypes (Table 1). The sick mice suffered from weight loss and exhibited signs of fatigue, hypoactivity, ruffled fur, paralysis of the limbs and motor impairment, which was accentuated by exercise. Their heads were sinking and their backs were exaggeratedly humped. Severely sick animals died from the disease, whereas in some cases the disease seemed to be transient.

Table 1 EAMG incidence and antibody titres against AChR in different strains of mice

Strain	H-2 Haplotype	log ₂ Haemagglutination titre			Animals with clinical signs of EAMG
		1 °C day 21	1 °C day 63	2 °C day 8	
A/J	a	8.2	13.5	20.3	1/5
B10.A	a	7.5	11.2	20.0	2/5
C57BL/6	b	8.0	11.8	18.9	8/12
C3H.SW	b	7.5	11.0	19.3	2/5
CWB	b	7.5	11.5	19.6	2/10
BALB/c	d	7.5	11.2	19.8	2/5
DBA/2	d	8.0	11.0	20.2	2/6
C3H/Hej	k	7.5	12.0	18.5	2/5
CKB	k	7.6	11.3	20.0	2/6
AKR/Cu	k	7.8	11.4	19.0	6/6
DBA/1	q	7.2	11.3	20.2	0/6
SWR	q	8.2	11.4	20.6	0/6
SJL/J	s	8.0	11.8	21.0	0/6
ASW	s	7.3	11.7	20.6	0/6

Antibody titres were determined by micropassive-haemagglutination technique. Formalinised sheep red blood cells were coated with *Torpedo* AChR (100 µg per ml packed cells) by means of tannic acid.

The symptoms of mice with EAMG were reversed temporarily soon after intravenous injection of 5 µg of edrophonium chloride (Tensilon). In particular there was improvement of motor performance such as gripping and walking. Further pharmacological evidence for a physiological aberration in neuromuscular transmission in mice with EAMG was obtained from a curare ((+)-tubocurarine) test¹⁴. All the AChR-injected mice of strains in which symptoms of EAMG were observed, including individual mice which did not exhibit symptoms, had a higher sensitivity to curare than did the resistant strains. For example, injection of 0.6 nmol of curare intravenously was lethal for the AChR-injected C57BL/6 mice, tested 5 months after the first injection, whereas 2.4 nmol was required to kill

Table 2 Incorporation of tritiated thymidine by cultures of lymph node cells from C57BL/6 mice sensitised with AChR

Antigen added to sensitised lymph node cells (µg per culture)		Stimulation index*	
		day 10	day 42
AChR	(0.50)	9.24	5.16
	(0.25)	10.07	7.14
Mouse (C57BL/6) muscle extract	(5.0)	4.30	Not done
	(2.5)	2.58	Not done
Rabbit muscle extract	(5.0)	6.50	8.66
Lysozyme	(2.0)	1.16	0.88

Microculture lymphocyte transformation technique was used according to the method described by Oppenheim *et al.*¹⁵. Mice were injected with 10 µg of AChR in CFA in the footpads 10 or 42 d before the experiments.

*Ratio of c.p.m. of experimental to control cultures; average of five experiments.

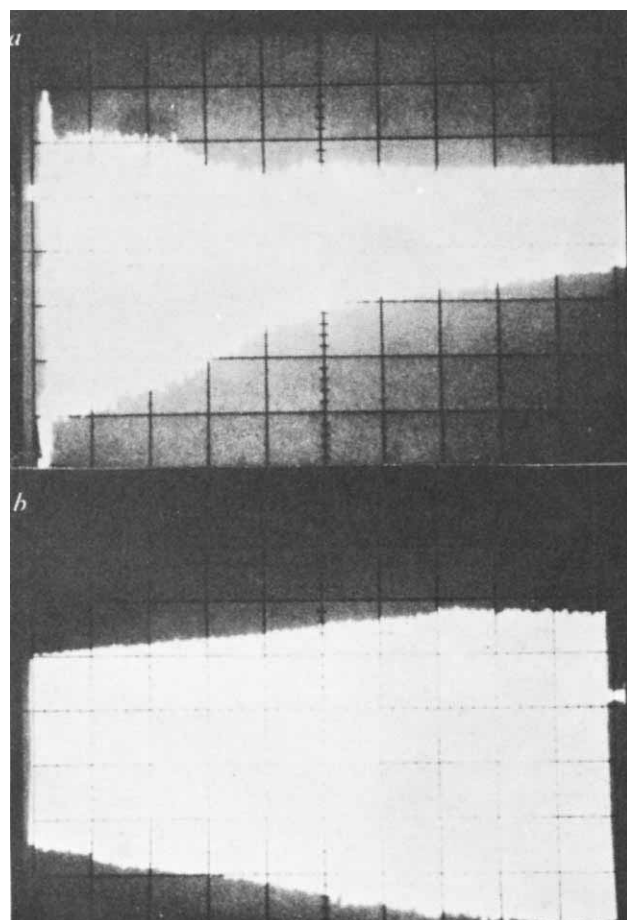


Fig. 1 Electromyographs from myasthenic and normal C57BL/6 mice. Supramaximal tetanic stimulation was applied to the sciatic nerve at a frequency of 20 per s with rectangular pulses of 0.1 ms duration and 0.75–1.5 V amplitude. The evoked compound muscle action potential was recorded with a coaxial needle electrode embedded in the calf muscle by an HP electromyograph. *a*, Myasthenic mouse; *b*, control mouse.

Table 3 Antibody response to AChR in an adoptive cell transfer experiment in C57BL/6 mice

Group no.	Cells transferred into lethally-irradiated mice	AChR injected	log ₂ Haemagglutination titre*		
			day 7†	day 12	day 18
(1)	Normal spleen cells (30 × 10 ⁶)	10 µg in CFA	2.25	5.0	6.37
(2)	Normal spleen cells treated with ATS‡ (30 × 10 ⁶)	10 µg in CFA	1.20	1.66	1.75
(3)	Primed spleen cells§ (30 × 10 ⁶)	10 µg in CFA	ND	13.0	14.75
(4)	Primed spleen cells treated with ATS (30 × 10 ⁶)	10 µg in CFA	ND	1.70	1.88

Cell-transfer experiments into lethally irradiated mice (800 rad ⁶⁰Co whole-body irradiation) were performed in a procedure similar to that described by McDevitt and Tyan¹⁷. ND, Not done.

*Average titre of 10–25 mice.

†Days after injection of AChR.

‡Anti-thymocytic serum.

§Cells obtained from mice injected with 10 µg of AChR in CFA 3 weeks earlier.

normal C57BL/6 mice. On the other hand, AChR-injected SWR and DBA/1 mice (H-2^b) as well as SJL/J and ASW mice (H-2^s) which showed no symptoms were not significantly more sensitive to curare than normal siblings which had not received antigen. Neurophysiological studies of C57BL/6 mice, made with an HP electromyograph revealed a decrease in amplitude during repetitive nerve stimulation in sick mice (Fig. 1a) and a normal response in control animals (Fig. 1b). Moreover, AChR-injected C57BL/6 mice which had no symptoms also showed a decremental response to repetitive nerve stimulation. The decremental electromyographic response as well as the pharmacological effects of Tension and curare are common criteria for verifying the neuromuscular block in MG.

The autoimmune response against self-AChR in C57BL/6 mice injected with *Torpedo* AChR was demonstrated by cellular and humoral reactivity towards syngeneic muscle extracts. Cellular sensitivity was tested by the *in vitro* lymphocyte transformation technique. As Table 2 shows, lymph node cells of mice injected once with *Torpedo* AChR were stimulated *in vitro* when incubated with the immunogen, as well as with xenogeneic (rabbit) and syngeneic (C57BL/6) muscle extracts. Humoral autoimmune response was demonstrated by the ability of mouse anti-AChR sera to bind to syngeneic AChR. An AChR-rich fraction was prepared from C57BL/6 mouse muscle, by solubilisation with Triton in a procedure similar to that described by Lindstrom *et al.*¹⁸ for the preparation of rat muscle extract. AChR in the extract was labelled with ¹²⁵I-bungarotoxin and the binding of labelled receptor to mouse antiserum was measured by radioimmunoassay. Sera from C57BL/6 mice immunised with *Torpedo* AChR bound, per ml of serum, an amount of mouse AChR corresponding to 0.35 pmol of toxin-binding sites.

Humoral immune response towards *Torpedo* AChR in the injected mice was determined using the micropassive-haemagglutination technique (Table 1). Significant titres of antibodies against AChR were observed in the mouse sera 3 weeks after a single immunisation. The antibody levels increased during the following 6 weeks and an additional significant increase was observed 8 d after the booster. No correlation between the antibody titres and incidence of the disease was found, as mice of all tested strains gave similar antibody titres (Table 1). It is possible, however, that there are differences in the specificity of the antibodies, especially as AChR is a high molecular-weight immunogen which presumably contains multiple potential immunopotential determinants. The susceptibility to EAMG may thus be determined by a genetically controlled ability to respond to a specific determinant (or determinants) not necessarily identical with the immunogenic determinants. Alternatively, circulating anti-AChR may not have a major role in the pathogenesis of EAMG. The mouse model disease which has a great advantage because of the availability of many inbred strains, together with immunochemical analysis of the AChR molecule can be helpful in the elucidation of this question.

The humoral response against AChR is T-cell dependent as was shown in adoptive transfer experiments (Table 3). It seems that T cells are required both for a primary and secondary immune response. Lennon *et al.*¹⁸ have previously demonstrated in rats that thymus-derived lymphocytes are required for the induction of EAMG and antibody to AChR. It should be interesting to try to dissociate the role of the thymus as a source of T-helping and T-suppressing cells from the possible specific role of the thymus as an antigenically cross reacting target with AChR¹⁹.

We have demonstrated the induction of EAMG in mice after two injections of *Torpedo californica* AChR. The clinical, pharmacological, electrophysiological and immunological findings in mice with EAMG closely parallel those in patients suffering from MG as well as in other animals with EAMG. Mice with EAMG were shown to react with self-AChR, probably as a result of breakdown of tolerance. Strains seem to vary in their susceptibility to the experimental disease. We do not know, however, whether there is a similar pattern for susceptibility to spontaneous disease, and whether such a pattern is genetically controlled.

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Selective IgA deficiency in chickens with spontaneous autoimmune thyroiditis

SELECTIVE IgA deficiency is believed to be the commonest form of human immunodeficiency disease, and occurs with a frequency of between 1: 500 and 1: 700 in random populations^{1,2}. Although the isolated deficiency is often considered non-pathological, there is clinical and laboratory evidence that IgA-deficient individuals suffer more sinopulmonary infection and autoimmune and gastrointestinal disorders^{3,4}. Family studies have suggested a genetic factor but no defined inheritance patterns have been established^{4,5}. The chicken model has been used for a number of years in our laboratory to study the ontogeny of immunoglobulin class development⁶. Surgical bursectomy at hatching^{7,8}, treatment of chick embryos with specific antisera^{7,9-11} and combined neonatal bursectomy-thymectomy¹² can result in depressed levels of IgA. This report describes recent observations of a selective IgA-deficiency that spontaneously occurs in the Obese strain (OS) of White Leghorn chickens. The OS chickens develop spontaneous autoimmune thyroiditis (SAT) at several weeks of age¹³. Clinical hypothyroidism is

been reported that selective IgA deficiency in humans is often associated with increased amounts of serum IgM as well as increased number of plasma cells containing IgM in the lamina propria of the small intestine, indicating that a compensatory mechanism may occur in some individuals⁵.

The relationship of autoimmune disease in humans to selective IgA deficiency has not been determined. While the majority of patients with autoimmune disorders have normal levels of IgA, those patients with the IgA deficiency have an increased incidence of abnormal κ/λ ratio, elevated levels of IgG and/or IgM, and serum 7S IgM⁴. Furthermore, those individuals who lack IgA have normal or increased numbers of IgA-bearing lymphocytes in the peripheral blood²⁰. These observations suggest that the abnormality in the immune response occurs in the latter stages of cell maturation and results in the absence of IgA-secreting plasma cells. With the advent of an animal model for spontaneous IgA deficiency it is now possible to study the genetic and cellular events responsible for this deficiency. Indeed, preliminary experiments in our laboratory have already suggested a genetic relationship between IgA deficiency and alleles at the major histocompatibility locus in chickens.

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Table 1 Serum immunoglobulin concentrations in OS and CS chickens*

Line	Age	Number tested	IgY(mg %)† ±s.d.	IgM(mg %)† ±s.d.	IgA(mg %)† ±s.d.	Number with undetectable IgA	Total number with < 10 mg % IgA
OS	6 weeks	12	228±2.9	146±1.3	5.57±3.9	4	7
CS	6 weeks	18	254±2.0	93.4±1.3	24.5±1.3	0	0
OS	1 yr	30	456±1.7	201±1.5	16.5±4.4	5	8
CS	1 yr	24	599±1.3	89.3±2.2	27.6±2.1	0	1

*Minimum sensitivity of the radial immunodiffusion assay is 2.0 mg % for IgY and 3.0 mg % for IgM and IgA.

†Serum immunoglobulin levels are presented as geometric mean. Analyses were performed on log₁₀ transformed data.

observed and high titres of circulating as well as bound anti-thyroglobulin antibodies can be detected¹⁴. The OS chickens were initially derived from the Cornell C-strain which reveals a 1% incidence of SAT. Selective breeding of CS birds, which showed phenotypic symptoms of hypothyroidism, resulted in the OS of which now, in generations 16 and 17, show 96% SAT. The histological and serological features in OS chickens make it a reasonable animal model to study Hashimoto's thyroiditis, an autoimmune disorder in humans¹⁵.

Serum immunoglobulins IgY (ref. 16), IgM, and IgA were quantified by radial immunodiffusion in Agarose gel with heavy chain-specific antisera and reference standards prepared in our laboratory¹⁷. This technique is conventionally used to diagnose selective IgA deficiency in humans¹⁸. Both systems can detect IgA concentrations as low as 2–3 mg %.

In Table 1 the serum immunoglobulin concentrations of a random population of 6-week and 1-yr-old OS and CS chickens are presented. Serum IgA concentrations were markedly decreased in OS chickens with a mean concentration of 5.6 mg % in 6-week-old birds and 16.5 mg % in 1-yr-old birds. Although many of these OS birds had normal levels of IgA, 33% (4 of 12) 6-week-old birds and 16% (5 of 30) 1-yr-old birds had undetectable levels of IgA (<3.0 mg %). In contrast, only one CS bird had an IgA level of less than 10 mg % and none had undetectable levels of IgA. In addition to the isolated IgA deficiency in OS chickens, an increase in serum IgM levels were noted. It was observed, however, that the IgA-deficient birds did not necessarily have a selective increase in IgM, but rather the increased IgM was present throughout the entire OS population. This is consistent with the increased number of IgM-bearing B-cells found in OS chickens¹⁹. It has previously

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Transformation of human lymphocytes by Epstein-Barr virus is inhibited by phosphonoacetic acid

PHOSPHONOACETIC acid (PAA) is an inhibitor of herpes simplex virus and of several other herpes viruses. It acts as a specific inhibitor of two viral DNA polymerases, those of herpes simplex and of Marek's disease (a herpes-induced leukaemia in chickens)^{1,2}. We have used PAA as a probe to investigate the possible existence of a virus-directed DNA polymerase and its role in replication of viral genomes during the transformation and proliferation of normal human peripheral lymphocytes induced by Epstein-Barr virus (EBV), which is a herpes virus closely associated with the development of several neoplasms in man.

Synthesis of the EBV capsid antigen (VCA), but not of the early antigen (EA), is dependent on DNA synthesis³. Moreover, EBV producer lines cease synthesis of VCA and biologically active virus after several days of treatment with PAA at concentrations which do not have a measurable effect on cellular growth, cellular DNA synthesis or expression of either EA or the EBV nuclear antigen (EBNA)^{4,5}. The average number of copies of the viral genome per cell also decreases in these conditions because of the inhibition of productive viral DNA synthesis. Replication of latent viral DNA seems to be unaffected by PAA^{5,6}. Although no EBV-specific DNA polymerase has been isolated, these data suggest the existence of such an enzyme.

Little is known about the events by which EBV causes resting normal peripheral lymphocytes to transform and proliferate. The only two indicators of transformation are an increase in cellular DNA synthesis^{7,8} and the appearance of EBNA several days after infection⁹. Because of the limitations of material and available techniques, information about the behaviour of the viral DNA during infection is limited. Replication of the viral DNA after infection is suggested, however, by the presence of multiple copies of the viral genome in lymphoblastoid cell lines produced by *in vitro* infection with EBV¹⁰.

EBV transforms only the B-cell population of peripheral blood lymphocytes. Therefore, purified B-cell populations from normal adults were used to study DNA synthesis after infection with EBV. Use of purified B cells greatly decreases the background level of DNA synthesis by untransformed cells and provides a much more sensitive measure of virus-induced DNA synthesis than does the use of unfractionated cells. This method is at least as sensitive as the more commonly used cord blood lymphocytes and a full account will be published elsewhere. Briefly, blood was drawn from normal healthy adult donors into heparinised syringes and mixed 1:1 with Hanks' balanced salt solution. The peripheral lymphocytes were purified by centrifuging over Ficoll-Hypaque¹¹. The lymphocytes at the interface were washed with medium (RPMI 1640 plus penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), glutamine (300 µg ml⁻¹) and 5% foetal calf serum) and passed over an immunoabsorbent column of goat (anti-human Fab') antibody coupled to Sephadex G-200 (ref. 12). The B-cell population was bound to the column and was eluted with a 1% solution of human γ globulin and then twice washed with medium. This population is > 95% B cells as judged by labelling with fluorescent rabbit (anti-human Fab') antibody.

The rate of DNA synthesis by normal peripheral B lymphocytes cultured in the presence of various concentrations of PAA and assayed 6–7 d after addition of the transforming strain of EBV from the supernatant of the B95-8 marmoset lymphoblast culture is shown in Fig. 1.

There was demonstrable inhibition of DNA synthesis with as little as 12.5 µg ml⁻¹, with 50% inhibition at 100 µg ml⁻¹ and complete inhibition at 200 µg ml⁻¹. As a control, the effect of PAA on DNA synthesis in B lymphocytes stimulated by phytohaemagglutinin or pokeweed mitogen was studied. There was no inhibition up to a PAA concentration of 100 µg ml⁻¹ and only 30% inhibition at 200 µg ml⁻¹.

Consistent with the above observations of DNA synthesis, PAA at 100–200 µg ml⁻¹ was sufficient to inhibit completely the outgrowth of B-cell cultures infected with the B95-8 strain and observed for up to 1 month after infection, compared with controls which grew out after 12 d (Fig. 2).

The inhibitor did not act directly on the virus, however,

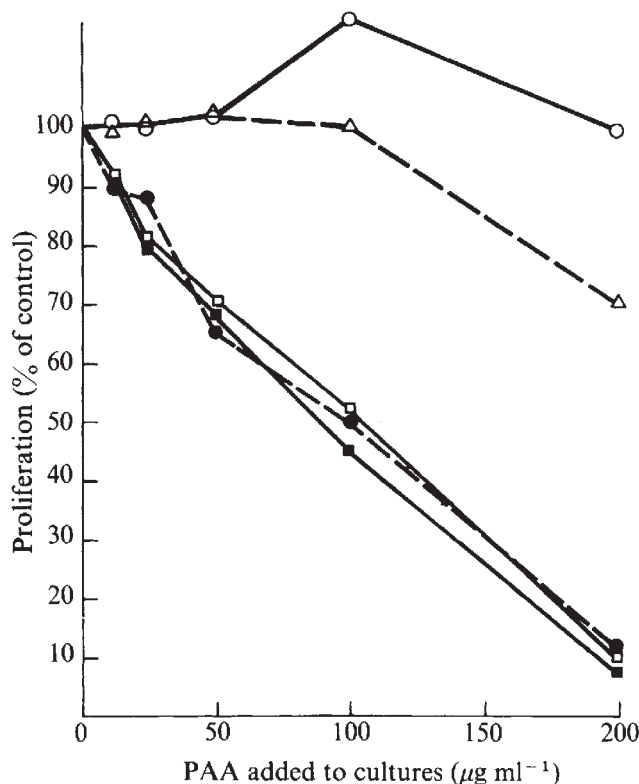


Fig. 1 Effect of PAA on DNA synthesis of normal peripheral B lymphocytes transformed by EBV or mitogens. Purified B cells were suspended at 2×10^6 ml⁻¹ in RPMI 1640 medium supplemented with 20% foetal calf serum, and 0.1-ml samples were added to Wells of a Linbro Micro Test II plate. EBV (see below), PHA (Burroughs Wellcome final concentration 0.25 µg ml⁻¹) and pokeweed mitogen (Gibco, final concentration 40 µg ml⁻¹) were diluted appropriately with the same medium and added in 0.1 ml to make a final volume of 0.2 ml per well. PAA, at appropriate dilutions, was added when required in a volume of 3–5 µl by means of an automatic delivery Hamilton syringe. All experiments were set up in triplicate. The cells were incubated at 37 °C in a humidified, 5% CO₂ atmosphere. After 6 d the cells were pulsed overnight with 50 µl of medium containing ³H-thymidine (New England Nuclear, 2 Ci mmol⁻¹, 2 µCi ml⁻¹). The cells were collected on glass fibre filters, washed repeatedly with saline followed by 5% ice-cold trichloroacetic acid and 95% ice-cold ethanol. The filters were dried and the remaining radioactivity was assessed by addition of 5 ml of Liquifluor scintillation fluid and counting. The experimental results are expressed as

$$\frac{\text{c.p.m. in experimental}}{\text{c.p.m. in positive control}} \times 100\%$$

The c.p.m. in the positive controls (EBV or mitogen-added but no PAA) were EBV 1:10 (●)=6,810; 1:50 (□)=6,490; 1:100 (■)=1,510; PHA (○)=6,764; pokeweed mitogen (△)=4,774. Broken and unbroken lines represent B cells from different individuals with unstimulated incorporations of 105 and 141 c.p.m. respectively. The B95-8 EBV concentrates used in these experiments were provided by Dr K. A. Traul of Pfizer Inc. lot no. 3373-11, TCID₅₀ 10^{6.5} U ml⁻¹ by induction of EBNA in cord blood, electron microscopic count 4×10^8 viral particles per ml.

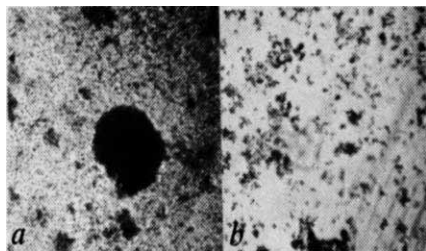


Fig. 2 Appearance of cultures infected with EBV to which PAA had not (*a*) or had (*b*) been added. Parallel cultures were infected with a supernatant from the B95-8 marmoset cell line, at a dilution of 1:10. Every 3–4 d half the culture medium was replaced with fresh medium. For full experimental details, see legend to Fig. 1*a*. Control culture infected with EBV alone and photographed 12–15 d after infection. After this time it is necessary to transfer the cells to larger wells to maintain them. Growth, as illustrated, occurred in all of 33 wells to which virus was added. *b*, Culture infected with EBV and maintained in the presence of PAA $100 \mu\text{g ml}^{-1}$ photographed 30 d after infection. At no time was an increase in cell number observed in any of 33 wells.

for preincubation with PAA for up to 6 h at 37°C did not affect the activity of the virus in stimulating DNA synthesis after removal of the inhibitor (data not shown). Furthermore, expression of at least two viral functions was unaffected insofar as PAA up to $200 \mu\text{g ml}^{-1}$ did not affect induction of EBNA in the peripheral B lymphocytes, nor did it affect induction of EA in the Raji cell line by the superinfecting strain of EBV from the P3HR-1 cell line (Table 1). Although PAA does not inhibit induction of EBNA in either adult peripheral B lymphocytes or those from cord blood, it is inhibited by cytosine arabinoside which blocks all DNA synthesis (I. Ernberg and G. Klein, personal communication). Both PAA and cytosine arabinoside are ineffective, however, in preventing EBNA induction during conversion of the EBV-negative lymphoblastoid line Ramos

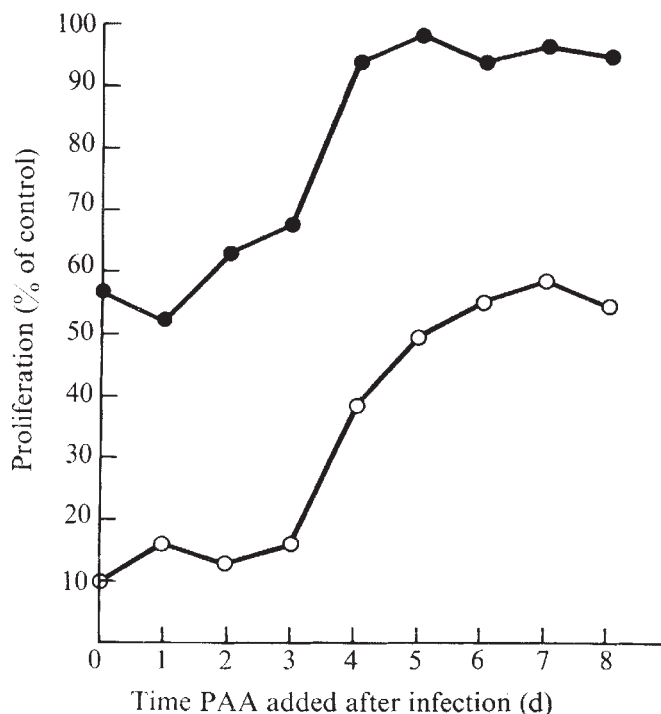


Fig. 3 Effect of PAA, added at various times after infection, on the rate of EBV-stimulated DNA synthesis. Purified human peripheral B lymphocytes were set up in culture in the presence of a 1:10 dilution of EBV as described in the legend to Fig. 1. PAA was added at various times in a volume of 3–5 μl to give the appropriate final concentration from an automatic delivery Hamilton syringe. The cells were assayed for DNA synthesis, as described in the legend to Fig. 1, 9 d after infection. ●, PAA final concentration $50 \mu\text{g ml}^{-1}$; ○, PAA concentration $200 \mu\text{g ml}^{-1}$. The c.p.m. in positive control = 1,976.

into its EBV-positive derivatives¹³. This suggests that cytosine arabinoside does not act directly on EBNA production but is effective in the transformation experiments because it prevents blastogenesis, and that once the cells have undergone blastogenesis, EBNA may be induced without DNA synthesis.

It is interesting that with PAA at $100\text{--}200 \mu\text{g ml}^{-1}$, cellular DNA synthesis stimulated by mitogens and EBNA induction were unaffected, whereas the transformation and outgrowth of EBV-infected cultures was inhibited. This implies that induction of EBNA is not sufficient to cause

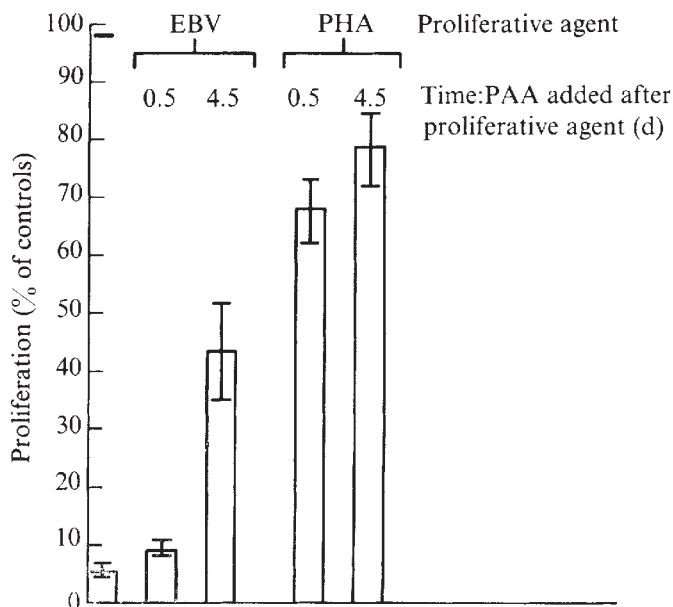


Fig. 4 Effect of adding PAA ($200 \mu\text{g ml}^{-1}$) 0.5 and 4.5 d after stimulation of DNA synthesis by EBV or PHA. Normal peripheral B lymphocytes were treated with EBV (dilution 1:10) or PHA as described in the legend to Fig. 1. PAA was added in 3–5 μl either 0.5 d or 4.5 d after cultures were set up. The rate of DNA synthesis was assayed 6 d after cultures were infected. Each column is the result of averaging the results obtained using cells from six different donors and the bars represent 1 s.d. The positive controls varied between 700 and 3,700 c.p.m. with EBV and 2,200 to 2,600 with PHA. A culture not treated with virus is shown in the left column.

cells to proliferate logarithmically even when the cellular DNA synthetic system is intact.

The nature of inhibition of DNA synthesis by PAA after EBV infection was studied further by adding the inhibitor to different cultures at various times after the virus. The rate of DNA synthesis was measured 9 d after infection. PAA was effective when added up to 3 d after infection but thereafter effectiveness decreased (Fig. 3). The effectiveness of PAA when added many hours after the virus confirms the observation that PAA does not act on the viral particle directly and implies also that it does not affect penetration of the cell by the virus. The decrease in effectiveness of PAA could have been a result of its slow incorporation; it took about 5 d to reach effective levels. This was ruled out, however, by the observation that the decrease in sensitivity occurred at the same time whether the cells were collected for assay 7 or 9 d after infection.

The decrease in the sensitivity of EBV-stimulated DNA synthesis to PAA is specific, as shown when PAA at $200 \mu\text{g ml}^{-1}$ was added to cultures 0.5 or 4.5 d after infection with EBV or stimulation with PHA (Fig. 4). There was little change in the sensitivity of PHA-stimulated cellular DNA synthesis with various times of addition, synthesis being inhibited by 20–30% in both cases, perhaps because of

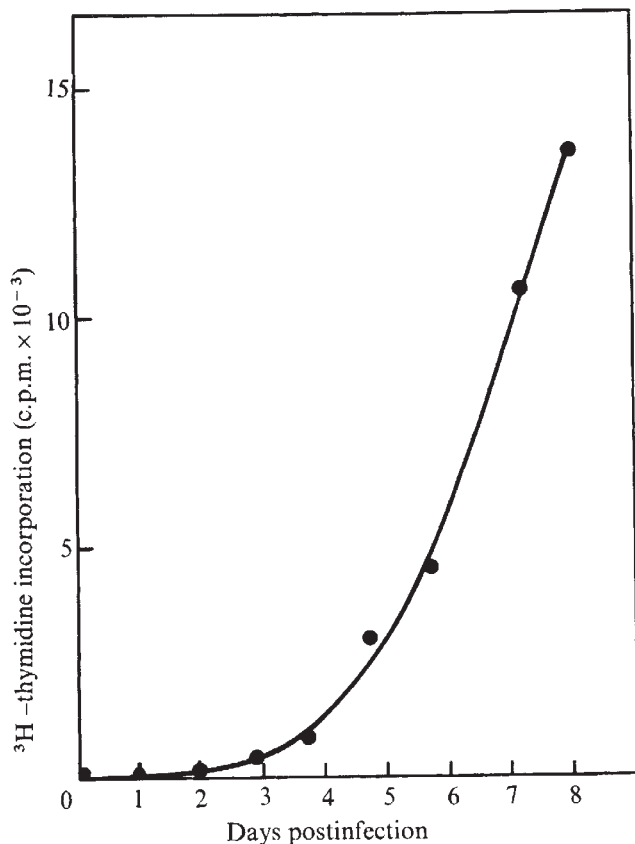


Fig. 5 Time course of DNA synthesis in lymphocytes infected with EBV. Parallel cultures of uninfected and virus-infected (by additions of supernatant solution from a culture of B95-8 cells diluted 1:10) cells were set up in triplicate at various times. Thereafter, the rate of DNA synthesis was assayed as before, except the cells were pulsed for 4 h. The specific rate of DNA synthesis was calculated by subtracting the value measured for the uninfected cultures from that obtained with the infected cultures. For full experimental details, see legend to Fig. 1.

inhibition of a host DNA polymerase which is reported to be sensitive to PAA (J. Liversidge and W. Benz, personal communication). The EBV-stimulated DNA synthesis was completely inhibited when PAA was added early after infection but only about 50% inhibited when added at 4.5 d.

The most likely explanation of these results is that there is a process which is sensitive to PAA and is required for the initiation of cellular proliferation. This process occurs during the first 3–4 d after infection, at which time cellular DNA synthesis begins and apparent PAA sensitivity decreases. This hypothesis is supported by the observation that a considerable increase in cellular DNA synthesis occurs at 3–4 d after EBV infection (Fig. 5), precisely the time after which addition of PAA is no longer effective in inhibiting transformation.

In conclusion, the data presented here indicate the existence of a PAA-sensitive event, which is necessary for transformation and unlimited proliferation of normal peripheral B lymphocytes. PAA does not, however, act directly on the virion nor does it block penetration of the cell by the virus or the subsequent expression of EBNA. It is known that cell lines produced by *in vitro* infection with EBV contain 10–15 copies of the viral genome per cell¹⁰. It seems likely therefore that some amplification of the viral genome occurs after infection. This amplification must cease after a short time and the viral genome number

Table 1 Effect of PAA on induction of EBNA in normal peripheral B lymphocytes and EA in the Raji lymphoblastoid cell line

	no virus	0	(PAA) $\mu\text{g ml}^{-1}$	200
EBNA+ve cells	—	+	ND	+
% EA+ve cells*	<0.25	5.1	5.8	4.8

To measure EBNA induction, 5×10^6 – 10×10^6 B lymphocytes were infected with B95-8 virus in the usual way (see legend to Fig. 1); 3–4 d later the cells were collected and stained for EBNA by the anticomplement immunofluorescent technique¹⁴. EA induction by P3HR-1 EBV in the Raji cell line was tested as described by Klein *et al.*¹⁵. Anti-EA antisera for the direct fluorescence test were provided by Dr G. Klein.

*Average of duplicates, about 2,000 cells each were counted.

ND, Not done; +, the same level of positive cells as in the control, approximately 5%.

is then kept constant—it replicates with the cellular DNA. The amplification may conceivably be a prerequisite for integration of one or more EBV genomes into the cellular DNA synthetic system, functionally and perhaps also physically. Alternatively, replication could be required simply for the transcription and translation of one or more genes required for transformation.

If a PAA-sensitive, virally induced DNA polymerase (by analogy with herpes simplex¹ and the Marek's disease² DNA polymerase) is required for gene amplification before integration, then it follows that viral DNA synthesis is required for true cellular proliferation. At the simplest level, the single cell infected with EBV in the presence of PAA may be transformed, able to express EBNA and to divide indefinitely. It carries only one non-replicating copy of the viral genome, however, and therefore can generate only one daughter cell carrying and expressing the viral genome and able to divide. Thus, cultures infected with EBV in the presence of PAA may contain transformed and dividing cells, but the cell number per culture would not increase exponentially and inhibition of proliferation would be observed.

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Complement-dependent adherence of mast cells to schistosomula

MAST cells are thought to have a major role in the host response to parasitic helminths. Infection with these organisms is often accompanied by anaphylactic hypersensitivity and mast cell infiltration¹. In several experimental models, host resistance to helminth infection has been shown to be dramatically suppressed as a result of mast cell depletion¹. For example, after injection with reserpine, laboratory mice are unable to mount a cutaneous inflammatory response against *Schistosoma mansoni* (A.S.,

P. W. Askenase, S. McIntyre and F. von Lichtenberg, unpublished) and after pretreatment with compound 48/80 can no longer immunologically reject challenge infections of this parasite³. Interactions between mast cells and helminths have generally been attributed to soluble antigens released into tissue fluids. Described here is a series of *in vitro* experiments with schistosomula of *S. mansoni* which demonstrates that mast cells are also capable of reacting directly with parasite surfaces. This interaction appears to depend on the recognition of parasite-bound complement by receptors on the mast-cell membrane.

Schistosomula were prepared by allowing *S. mansoni* cercariae to penetrate rat skin *in vitro*³, and were stored overnight at 4 °C before use⁴. Mast cells were purified from peritoneal washouts of uninfected male Charles River CD (Caesarean derived) rats (175–300 g) by centrifugation over Metrizamide (22.5%)⁵. The cells recovered from the pellet fraction were routinely found to contain a minimum of 90% mast cells as determined by toluidine-blue staining. Both schistosomula and mast cells were washed three times in HEPES-buffered Hanks' minimal essential medium (MEM) containing 1% heat-inactivated foetal calf serum (FCS) and resuspended in MEM containing 10% FCS (MEM/FCS). A serum pool obtained from CD rats 8 weeks after a primary infection⁶ of 1,500 cercariae each was used as a source of anti-schistosome antibody. The immune serum was diluted in MEM/FCS and used in the assay at a final concentration of 1/20. The adherence test was carried out

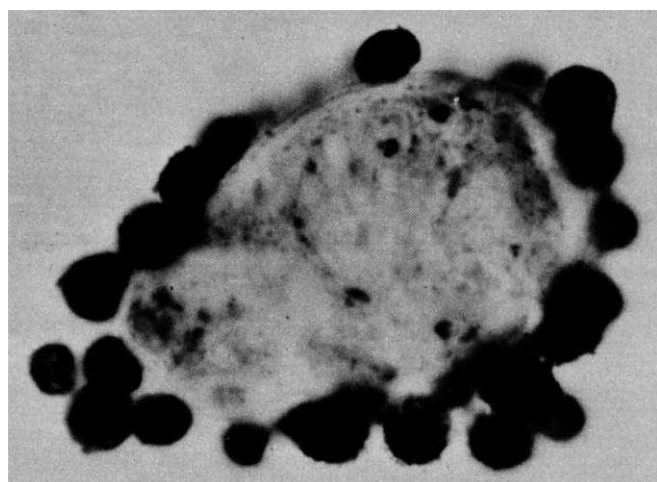


Fig. 1 Photomicrograph (×216 magnification) of a typical schistosomulum after incubation with fresh immune rat serum and purified rat mast cells.

in glass tubes (6 × 50 mm) containing the following sequential additions: 0.1 ml of schistosomula (90–110 organisms), 0.1 ml of diluted immune serum, 0.1 ml of diluted normal rat serum (complement source), and 0.1 of mast cells (2×10^5 – 4×10^6 total cells). After incubation at 37 °C for 90 min, the supernatant was withdrawn from each tube and the cell-parasite sediment resuspended in 0.1 ml of MEM/FCS containing 0.01% toluidine-blue. The parasites were then examined microscopically at 80 times magnification for adhering mast cells. Organisms with more than five adherent cells were scored as positive. Each result is the average of readings on replicate assay tubes scored independently by two different observers.

Schistosomula incubated with fresh immune rat serum formed 'rosettes' with the purified mast cells (Fig. 1), each positive organisms having a coat of about 20–100 adherent, toluidine-blue-positive cells. Approximately 90% (Table 1) of the larvae in the incubation mixture reacted in this fashion. In contrast, few if any mast cells adhered to schistosomula incubated in heat-inactivated (2 h, 56 °C) immune serum (Table 1). The addition of fresh normal rat serum to the heat-treated immune serum restored its full activity, an observation which suggested that the heat-labile factor was complement

Table 1 Adherence of rat mast cells to schistosomula in the presence of rat sera

Immune rat serum	Normal rat serum	Mast cell coated organisms (%)
Fresh	None	89
Inactive	None	14
Inactive	Fresh (1/20)	93
Inactive	Fresh (1/40)	80
Inactive	Fresh (1/80)	33
Inactive	1 h, 56 °C (1/20)	21
Inactive	30 min, 50 °C (1/20)	32
Inactive	Fresh (1/20) + EDTA (5×10^{-3} M)	3
Inactive	Fresh (1/20) + EGTA (5×10^{-3} M)	89
Inactive	Fresh (1/20) + Cobra factor	35
Inactive	+ Zymosan, 1 h, 37 °C (1/20)	8
Inactive	+ Zymosan, 2 h, 17 °C (1/20)	20
None	Fresh (1/20)—Pool A	46
None	Fresh (1/20)—Pool B	85
None	*Preincubation in Pool B (1/20)	85
None	*Preincubation in Pool B (1/20) + EDTA (5×10^{-3} M)	8
None	None	1

*Schistosomula preincubated with rat serum (1 h, 37 °C) and washed three times (with MEM/FCS) before addition of mast cells.

rather than antibody. This hypothesis was supported by a series of experiments (Table 1) in which the adherence-promoting activity of fresh normal rat serum was shown to be partially or completely abolished by the following treatments known to inactivate complement^{7,8}: (1) heating for 1 h at 56 °C; (2) preincubation with either zymosan (15 mg ml⁻¹ serum) or cobra venom factor (200 U ml⁻¹ serum) for 1 h, at 37 °C; (3) the addition of EDTA (5×10^{-3} M) to the cultures. The dependence of the adherence reaction on the alternate pathway of complement^{9,10} was suggested by the failure of EGTA (5×10^{-3} M)¹¹ to inhibit rosetting and by the reduction in activity observed after heating the normal serum at 50 °C for 30 min (ref. 9) or pretreating it with zymosan at low temperature (2 h, 17 °C)⁹.

Schistosomula incubated in normal rat serum without immune serum as an antibody source were also found to bind mast

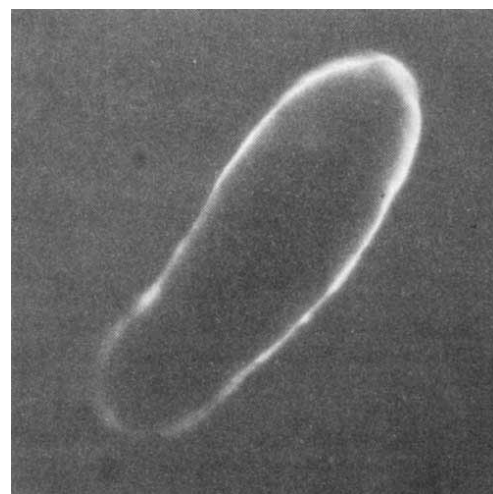


Fig. 2 Photomicrograph (×224) of a typical schistosomulum, preincubated with normal rat serum (Pool B), washed three times in MEM/FCS and stained with fluorescein-labelled rabbit antibody specific for rat C3 (from Cappel Laboratories). No evidence of staining was observed when the same organisms were treated with fluorescein-labelled rabbit antibody (Cappel) produced against rat IgG but cross reactive with rat immunoglobulins of other classes. In contrast, schistosomula preincubated with immune rat serum stained brightly (as above) with the same anti-immunoglobulin reagent.

cells, different serum pools inducing different levels of adherence (Table 1). After preincubation with normal rat serum (1 h, 37 °C) and extensive washing (with MEM/FCS), schistosomula remained highly reactive with mast cells. The activity of normal serum in promoting adherence did not seem to be due to the presence of 'natural antibodies' with specificity for schistosome surface antigens since membrane-bound rat antibodies were not detected on worms pretreated with normal serum by means of immunofluorescent staining with fluorescein-labelled antibody specific for rat immunoglobulins. In contrast the same pretreated organisms stained brightly (Fig. 2) with an immunofluorescent reagent specific for rat C3. Neither C3 staining nor mast-cell adherence was observed if EDTA (5×10^{-3} M) was added to the normal rat serum in the preincubation mixture.

These results suggest that the adherence of mast cells to schistosomula is dependent on the recognition of parasite-bound complement by receptors on the mast cell surface. Complement in a form suitable for recognition by mast cells is acquired by the worms either as a consequence of the reaction of antibody at the parasite's surface or, in the absence of antibody, by direct fixation. Previous studies have indicated that cercariae of *S. mansoni* possess an alternative pathway-dependent complement (C3)-activating system¹². The retention by schistosomula of a similar complement-activating mechanism after conversion from the cercarial stage of the life cycle would explain their behaviour in the experiments reported here.

An important question raised by the mast cell-schistosomulum adherence phenomenon concerns the nature of the receptors on the mast-cell surface involved in the recognition of the parasite-complement complex. Rat mast cells have been shown to respond to the anaphylatoxins, C3a and C5a¹³. Since both complement fragments are normally released in the fluid phase and are extremely labile¹³, it is unlikely that the adherence reaction involves receptors for these molecules. An alternative hypothesis is that mast cells, in common with platelets, primate erythrocytes, neutrophils, monocytes, macrophages and B lymphocytes, possess receptors for surface-bound activated C3 (refs 14, 15) and that the mast cell-schistosomulum interaction is dependent on this previously unrecognised set of immune adherence receptors.

Other questions raised by the adherence phenomenon concern the effects of the interaction on the release of mediators from mast cells and on the viability of the target organisms. Experiments aimed at answering these questions are now in progress.

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Variable Ca sensitivity of a K-selective channel in intact red-cell membranes

THE membranes of many cells exhibit a K-selective permeability mechanism with a gating process controlled by the concentration of Ca^{2+} in the cytosol¹. Originally described in human red cells², where its function still remains unknown, this or similar mechanisms³ seem to mediate important physiological actions in other cells⁴⁻⁸.

The human red blood cell remains, nevertheless, the best model for the study of the interaction between Ca and the K gating mechanism because the composition of the media on both sides of the membrane can be known and controlled better than in any other cell¹⁰⁻¹³. This control is achieved, however, by treatments which are known to alter considerably the Ca reactivity of Ca-transporting mechanisms¹⁴⁻¹⁶. We

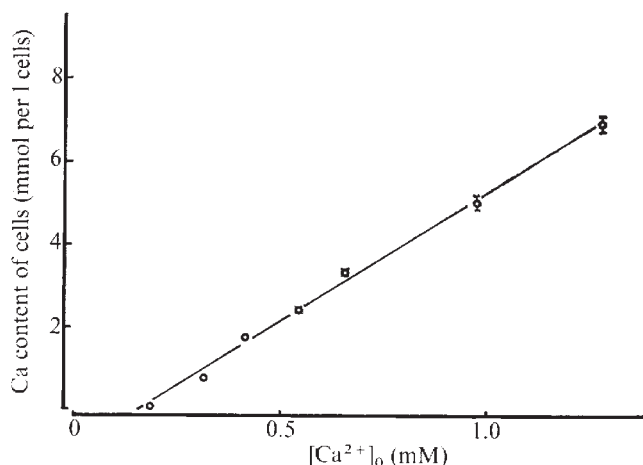


Fig. 1 Steady-state Ca content of intact red cells as a function of the external Ca^{2+} concentration in the presence of the divalent cation ionophore A23187. Red cells from fresh blood or from 3-4-d-old bank blood were washed as described previously¹⁸ and then resuspended at about 10% haematocrit in a medium containing KCl, 75 mM; Tris-HCl (pH 7.5 at 37 °C), 10 mM; NaCl, 75 mM; MgCl_2 , 1 mM; Inosine, 10 mM; 5 μCi of ^{45}Ca , and various concentrations of CaCl_2 up to about 2.5 mM. The total volume of suspension was 2.25 ml. At $t = 0$, 10 μl of absolute ethanol containing 0.05 to 1 mg ml^{-1} of the ionophore (0.1 mg ml^{-1} in this experiment) were added to the suspension which was in a magnetically stirred polythene vial inside a waterbath at 37 °C. At $t = 15.5$ min, 0.1 ml of isotonic $^{42}\text{K-KCl}$ was added to the suspension. At 15, 16, 16.5, 17, 18, 20, 25 and 30 min, 0.1 ml samples were placed in 1.5-ml Eppendorf centrifuge tubes containing 0.4 ml of *n*-butylphthalate (BDH, density 1.042-1.045) and 0.9 ml of an isotonic $[\text{Na}+\text{K}]$ -medium containing 5 mM Tris-EGTA, all at 0-1 °C. Each sample was centrifuged immediately for 10 s at 12,000g in an Eppendorf centrifuge model 3200. The cells formed a compact pellet at the bottom, retaining only about 0.1% of the original extracellular fluid as estimated using ^{45}Ca in the presence of a large excess of EGTA. The aqueous supernatant remaining on top of the oil layer was removed by aspiration and the walls of the tube cleaned with cotton swabs. The cells were lysed and the proteins precipitated with 5% trichloroacetic acid (TCA) all in the same tube. Aliquots of the TCA-supernatant were used for counting both isotopes in the same samples. ^{45}Ca was counted after ^{42}K decay. Samples for total activity and haemoglobin were taken at the end of each run. In the media used here, the haematocrit did not change during the experiments and the internal K concentration remained constant over a thousandfold change in K permeability at about 130 mmol per l cell water. The results concerning the ^{42}K fluxes are given in Fig. 2. The Ca content of the cells was calculated as the mean ± 2 s.e.m. of the seven time points. The slope of this particular curve is 5.91. The fraction of ionised Ca inside the cells, as calculated from this slope according to Ferreira and Lew¹⁸, was 0.33. This value was used to estimate the concentration of ionised Ca in the cytoplasm of the cells in the experiment of Fig. 3 and an identical procedure was applied in the experiment of Fig. 4.

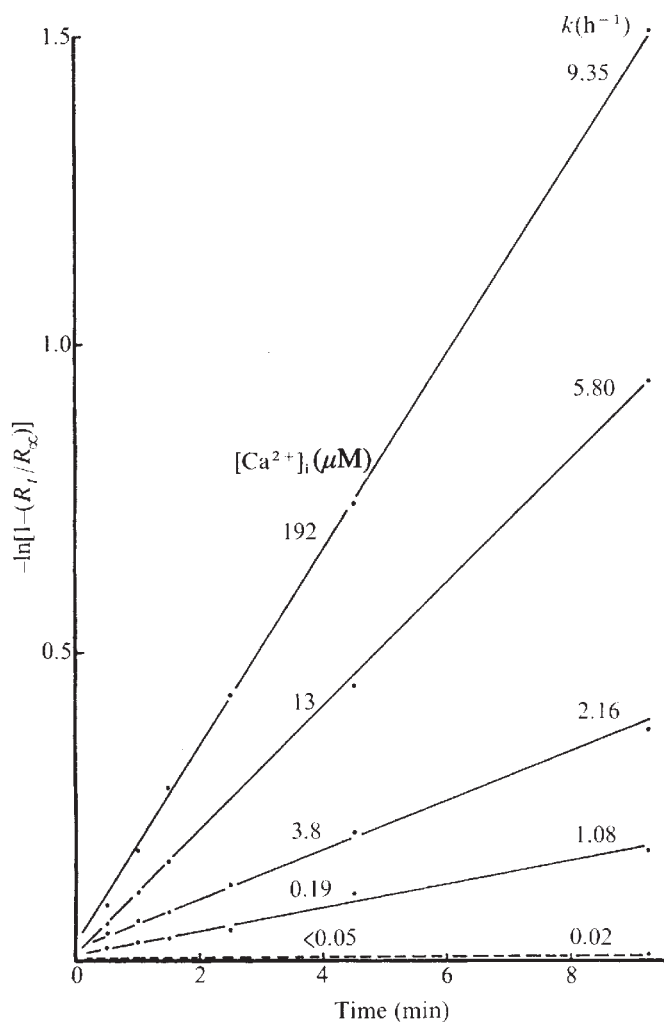


Fig. 2 The rate of tracer (^{42}K) equilibration in intact red cells at different intracellular levels of Ca^{2+} . The tracer equilibration process followed first-order kinetics and was adequately described by the equation

$$R_t = R_\infty(1 - \exp(-kt))$$

where R_t and R_∞ represent the activity of ^{42}K in identical volumes of cells at time t and at equilibrium and k is the rate constant of the equilibration process. This equation can be rearranged to give

$$-\ln[1 - (R_t/R_\infty)] = kt$$

The figure is a plot of $-\ln[1 - (R_t/R_\infty)]$ against t during some of the Ca steady states obtained in the experiment of Fig. 4 and is meant to illustrate the method used to calculate k . Since the normal volume-area ratio of the fed red cells remained unaltered during the present experiments, the K permeability (P_K) of the red-cell membrane, can be calculated from k . When $k = 1 \text{ h}^{-1}$, P_K will be between 1.5×10^{-8} and $2.1 \times 10^{-8} \text{ cm s}^{-1}$. The first numerical value given on each curve corresponds to the Ca^{2+} concentration inside the corresponding cells (in $\mu\text{mol per l cell water}$).

report here the surprising finding that in the intact, minimally disturbed red cell, Ca activates the K flux with a much lower affinity than that previously found in ATP-depleted red cells or resealed ghosts^{1,12,13}, suggesting that the higher Ca sensitivity of these systems results from treatment-induced alterations in the reactivity of labile Ca -interacting sites.

To incorporate controlled amounts of Ca inside the cells and to keep a known and constant level of ionised Ca during the measurement of the Ca -induced K flux, we used a divalent cation ionophore, A23187 (ref. 17), and a fast resolution technique for tracer flux measurements, which we developed recently¹⁸ to study cytoplasmic Ca buffering and Ca -pump parameters in intact cells.

Since the normal mammalian red cell is virtually free of Ca^{19} , is almost impermeable to $\text{Ca}^{15,16}$ and has a powerful Ca -extrusion pump in its membrane¹⁴, steady states with variable internal Ca concentrations could be obtained only as a balance between active Ca extrusion and ionophore-induced leaks (Fig. 1). The membrane potential was held constant by a 'chemical clamp' method in which the steady-state external concentrations of all permeable ions are in electrochemical equilibrium with the corresponding internal ions at a pre-determined potential, about -10 mV in the present experiments. At each ionophore concentration the level of internal Ca was regulated by varying the Ca concentration in the medium, and the fraction of ionised Ca was estimated from the slope of the curve relating the total internal Ca to the external Ca^{2+} concentration when the Ca pump was saturated (see text of Fig. 1). The influx of K was estimated by measuring the rate of ^{42}K -tracer equilibration at constant internal Ca and with the total external K concentration at electrochemical equilibrium with the internal K (Fig. 2). In every case, single exponentials were obtained with all the internal K participating in the equilibration process. In the absence of Ca , the ionophore had no effect on the Na and K permeability of the membrane. The entry of Ca induced a variable increase in the K flux while at the same time it reduced the Na efflux, presumably by inhibiting the Na pump¹. Quinine (1 mM) in the medium inhibited the Ca -dependent flux in the present experiments by about 95%, just as it inhibited the Ca -induced increase in K permeability in the absence of the ionophore³.

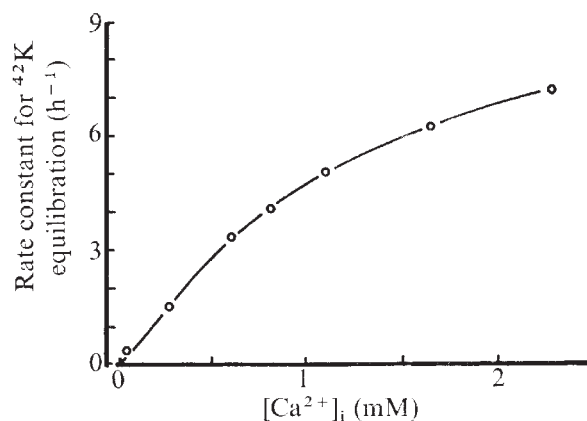


Fig. 3 The rate constant of tracer (^{42}K) equilibration as a function of the internal Ca^{2+} concentration in intact red cells in the presence of a low ionophore concentration. The internal Ca^{2+} concentration and k were obtained as indicated in the legend of Figs 1 and 2 respectively. The proportion of ionophore to cells was about $10 \mu\text{mol per l}$ of cells.

Figure 3 shows the variation in the rate constant of tracer (^{42}K) equilibration as a function of the internal concentration of Ca^{2+} in intact cells exposed to a relatively low ionophore concentration. The apparent K_m for Ca^{2+} is about 1.2 mM and varied in different experiments with similar ionophore concentrations between 0.33 and 1.5 mM . If the ionophore concentration is increased by ten times or more, the kinetics of Ca activation of the K permeability are completely different (Fig. 4) and approach the pattern previously observed in metabolically depleted resealed ghosts¹³. In ATP-depleted intact cells¹⁰ we always obtained high Ca affinity-type curves, whatever the ionophore concentration, but in such cells the maximum increase in K permeability was only half that of the same cells before depletion. All these different patterns were consistently reproducible.

The variation in Ca sensitivity cannot be attributed to Mg^{2+} since the distribution of this ion at equilibrium must have been the same in all the experiments. To investigate whether the high and low Ca affinity patterns were related to the ATP content

of the cells, we measured the intracellular levels of ATP in the various conditions in which we tested the Ca effect on the K fluxes. The addition of ionophore in the absence of Ca slightly increased the ATP content of the cells in a manner reminiscent of the effect of propranolol²⁰. The additional presence of Ca stimulated ATP hydrolysis through the Ca pump and induced a rapid fall in ATP from 1 mM to about 0.55 mM followed by a slower decline, presumably because of the buffering effect of membrane-bound adenylate kinase. These effects were independent of the ionophore concentration, which makes ATP alone an unlikely source of the different kinetic patterns observed in the intact cells. It is also unlikely that the variable Ca sensitivity of the K permeability system results from a non-homogeneous distribution of Ca within the cell since the K fluxes we report here were measured simultaneously in the same cells (see legend to Fig. 1 in ref. 18) where we had previously determined a constant K_m of 1 μ M for the Ca activation of the Ca pump at all ionophore concentrations.

We also investigated the effect of ionophore removal on the Ca-dependent K flux. This we could only try in intact red cells loaded with Ca if the Ca pump were inhibited, and for this reason we first depleted the cells of Mg, a necessary internal cofactor for Ca extrusion²¹. Mg extraction and Ca loading were performed in succession by preincubating the cells in the presence of ionophore (10 μ mol per l cells) first in a medium containing 2 mM EDTA and no Ca or Mg and then in Ca-containing media in the absence of Mg and chelator. The ionophore was then removed by washing the cells eight times in Ca-free ice-cold solutions at low haematocrits (about 1%). This simple procedure restored the normal Ca impermeability of the intact cell and, with the Ca pump arrested, the cells remained effectively resealed to Ca, losing only about 25% of their original Ca content during the initial washes. These cells also showed a high Ca affinity pattern similar to that of the intact cells at high ionophore concentrations shown in Fig. 4.

The low Ca affinity pattern shown in Fig. 3 has never been observed before. Since the cells were disturbed least in these conditions we feel that the low affinity response is closer to the behaviour of the normal system. The high affinity pattern would then correspond to an altered reactivity to internal Ca which may or may not occur in the normal cell depending on whether the Ca sensitivity of the K permeability mechanism is or is not subject to control by cellular regulatory mechanisms. Since the maximum increase in K permeability induced by Ca is more than three orders of magnitude larger than the "ground" K permeability of the intact red cell, an increase in Ca_i^{2+} even in the micromolar range²², may suffice to produce a functionally significant increase in K permeability. A relatively

small effect could thus account for the Ca-induced hyperpolarisation observed in amphiuma red cells²³ and perhaps also in other cells^{5,8}.

The heterogeneous behaviour of ATP-depleted red cell populations in relation to the Ca-induced K flux^{15,23} can now be interpreted as resulting from a variable Ca affinity of the K gate. This revives the old controversy¹⁰ about whether the depleting procedure accumulates²⁵ or removes²⁶ metabolites which control the K permeability of red-cell membranes, but now it can be reformulated more precisely in terms of the control of the Ca sensitivity of the K-gating mechanism.

Since many of the conditions in which the effect of internal Ca on the K permeability is obtained resemble those in which 1,2-diacylglycerol accumulates in the red-cell membrane, as recently shown by Allan and Michell²⁷, we feel tempted to suggest the possibility that 1,2-diacylglycerol, or a similar metabolite, mediates the Ca effects on the K channel. According to Allan and Michell, the rate of formation of this substance depends on the internal Ca^{2+} concentration, whereas the fast conversion to an inactive phosphatide, by the action of a diacylglycerol kinase, would depend on the intracellular level of ATP. If the Ca effect on the K permeability depends on the level of a certain intermediate, the different Ca sensitivities observed in the present experiments may merely reflect the variation in the relative rates of formation and breakdown of this intermediate in the different conditions.

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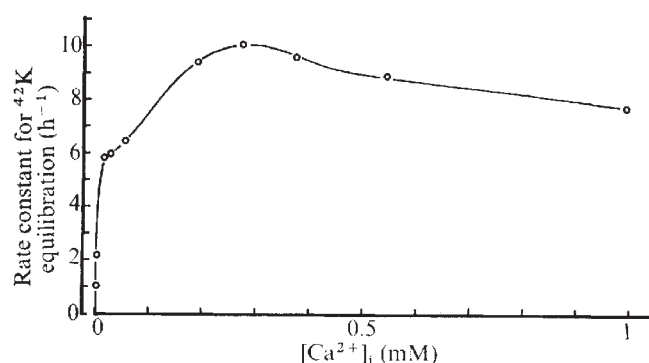
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Fig. 4 The rate of ^{42}K equilibration as a function of the internal Ca^{2+} concentration in intact red cells in the presence of a high ionophore concentration. The concentration of ionophore used in this experiment was 1 mg ml⁻¹ in the ethanol phase. This is equivalent to a proportion of ionophore to cells of about 100 μ mol per l of cells.



Stereospecificity of interaction of neuroleptic drugs with neurotransmitters and correlation with clinical potency

THE molecular mechanism responsible for the therapeutic activity of a drug is often suggested by the biochemical event which correlates best with clinical potency. Thus, if optical or geometrical isomers of a drug have markedly different clinical potencies, the most relevant biochemical effect should also exhibit isomeric specificity.

While numerous biochemical mechanisms have been proposed to explain the antischizophrenic action of neuroleptics, the

ability of these drugs to antagonise behavioural effects of apomorphine and amphetamine in animals correlates best with clinical potency¹. Since apomorphine and amphetamine-induced behaviours are mediated by brain catecholamines, especially dopamine, the neuroleptics are thought to act by blocking dopamine receptors in the brain. Clinical potencies of phenothiazine neuroleptics correlate reasonably well with their ability to inhibit a dopamine-sensitive adenylate cyclase which is presumably linked to the dopamine receptor^{2,3}. Because pharmacologically potent butyrophenones are relatively weak inhibitors of this cyclase and show only a partial correlation with clinical potency⁴, however, it has been suggested that these drugs may only indirectly affect dopamine receptors, perhaps by blocking dopamine release⁵. The chemical similarity of butyrophenones to γ -aminobutyric acid (GABA) suggests that these agents might in some way facilitate GABA transmission in the brain¹. Since inhibitory GABA neuronal pathways may synapse on dopamine cell bodies, facilitation of GABA effects could reduce the firing rate of dopamine neurones resulting in behaviours like those associated with dopamine receptor blockade⁶.

In both clinical and animal pharmacological screening, certain neuroleptics display isomeric specificity. α -Flupenthixol and *cis*-thiothixene are much more potent clinically and in animals than their geometrical isomers β -flupenthixol and *trans*-thiothixene respectively^{7,8}. Similarly (+)-butaclamol is much more potent than its optical isomer (–)-butaclamol⁹. The relative potencies of butaclamol, flupenthixol and thiothixene isomers upon the dopamine-sensitive adenylate cyclase accord with their isomeric specificity in clinical studies and pharmacological screens^{10,11}.

Using recently developed binding techniques to study neurotransmitter receptors directly, we have examined critically how reliably stereospecificity and correlations between clinical and biochemical effects can reveal the biochemical basis of neuroleptic drug action. The results suggest that although these criteria are valuable, they can obfuscate rather than clarify drug mechanisms if used incautiously. Of the several biochemical effects of neuroleptics, blockade of dopamine receptors best accounts for therapeutic effects of both butyrophenone and phenothiazine neuroleptics.

Synaptic receptor binding studies were conducted as previously described^{12–20}. Briefly, washed crude synaptic membrane or total particulate fractions of various brain regions representing approximately 1 mg of protein were incubated in a buffered solution containing a low concentration of tritiated ligand (1–8 nM) in the presence or absence of various concentrations of the drug under study. After incubation, radioactivity bound to the membranes was separated by filtration or

centrifugation, extracted into an appropriate fluor and counted by liquid scintillation spectrometry. The amounts bound in the presence and absence of the drug were compared. The ligands used in this study and the receptor to which each binds is as follows: ³H- γ -aminobutyric acid (³H-GABA), the GABA receptor¹²; ³H-dopamine (³H-DA and ³H-haloperidol (³H-Halo), the dopamine receptor^{13,14}; ³H-naloxone (³H-Nal), ³H-dihydromorphine (³H-DHM), the opiate receptor¹⁵; ³H-dihydroalprenolol (³H-DHA), the β -adrenergic receptor^{16,21}; ³H-5-hydroxytryptamine (³H-5-HT) and ³H-lysergic acid diethylamide (³H-LSD), the 5-HT receptor^{17,18}; ³H-quinuclidinyl benzilate (³H-QNB), the muscarinic cholinergic receptor¹⁹; and ³H-strychnine (³H-Stry), the glycine receptor²⁰.

Receptor binding for each ligand was assayed in the brain region containing the highest density of receptor sites or in whole brain. The highest density of dopamine receptor sites is in the corpus striatum^{13,14}. Binding of ³H-strychnine to glycine is most enriched in spinal cord membranes²⁰. Receptor binding for ³H-LSD, ³H-5-HT and ³H-DHA is greatest in the cerebral cortex^{16–18,21}. Although there exist regional variations in opiate, GABA and muscarinic receptor binding, the characteristics of binding sites do not vary regionally and substantial activity is present in whole brain or forebrain, which were used for assays of these receptors^{12,15,19}.

Uptake of radioactive GABA by synaptosomes in nuclei-free sucrose homogenates of whole rat brain was assayed as previously described²².

All radioactive agents were purchased from New England Nuclear, and all other substances were obtained from the company of origin.

There are no marked stereospecific influences of neuroleptics on receptor binding for ³H-GABA, for the opiates ³H-naloxone and ³H-dihydromorphine, for binding of ³H-strychnine to the glycine receptor, for the binding of ³H-quinuclidinyl benzilate (³H-QNB) to the muscarinic cholinergic receptors or for ³H-dihydroalprenolol (³H-DHA) binding to the β -noradrenaline receptor (Table 1). In contrast, the neuroleptic isomers are extremely potent in competing for ³H-haloperidol binding to the dopamine receptor and display striking stereospecificity. (+)-Butaclamol reduces ³H-haloperidol binding 50% at 1 nM and is more than 1,000 times as potent as (–)-butaclamol in eliciting this effect. α -Flupenthixol inhibits ³H-haloperidol binding 50% at 2 nM and is 50 times as potent as β -flupenthixol, while *cis*-thiothixene is 100 times more potent than *trans*-thiothixene in competing for ³H-haloperidol binding. These neuroleptics also demonstrate stereospecificity with respect to ³H-dopamine binding, although they are less potent in competing with this ligand. Thus, while (+)-butaclamol is less than 1% as potent in inhibiting ³H-dopamine as ³H-haloperidol

Table 1 Displacement of various receptor ligands by neuroleptic isomers

Compound	³ H-DHA ^a	³ H-5-HT ^b	³ H-LSD ^c	³ H-DA ^d	IC ₅₀ (μ M)*		³ H-Stry ^e	³ H-Nal ^f	³ H-DHM ^g	³ H-GABA ^h
					³ H-Halo ⁱ	³ H-QNB ^j				
(+)-Butaclamol	N.e. [†]	1.0	0.05	0.1	0.001	100	N.e.	18	17	N.e.
(–)-Butaclamol	N.e.	8.0	7.0	16.0	1.3	40	N.e.	20	17	N.e.
α -Flupenthixol	N.e.	4.0	0.10	0.2	0.002	0.7	N.e.	55	100	100
β -Flupenthixol	N.e.	60.0	6.0	10.0	0.10	0.9	N.e.	50	100	N.e.
<i>cis</i> -Thiothixene	80	1.0	0.3	0.7	0.003	3.2	50	15	30	50
<i>trans</i> -Thiothixene	80	7.0	1.0	19.0	0.30	2.8	50	15	35	50

*Concentration which inhibits ligand binding 50%.

[†]No effect at 100 μ M.

Four to six concentrations of each drug were evaluated in triplicate to obtain IC₅₀ values. Data are the mean of 2–4 determinations which varied less than 20%.

^aRat cerebral cortex membranes, ³H-DHA = 1 nM.

^bRat cerebral cortex membranes, ³H-5-HT = 7 nM.

^cRat cerebral cortex membranes, ³H-LSD = 3 nM.

^dCalf striatal membranes, ³H-DA = 5 nM.

^eCalf striatal membranes, ³H-Halo = 2 nM.

^fRat forebrain homogenate, ³H-QNB = 1 nM.

^gRat spinal cord-brainstem, synaptosomal membranes, ³H-Stry = 2 nM.

^hRat forebrain membranes, ³H-Nal = 1 nM.

ⁱRat forebrain membranes, ³H-DHM = 1 nM.

^jRat brain synaptosomal membranes, ³H-GABA = 8 nM.

Table 2 Inhibition of GABA uptake into rat brain synaptosomes by neuroleptic drugs

Compound	IC ₅₀ (μM)*	Average clinical daily dose† (μmol kg)
Butyrophenones		
Fluspirilene	3	0.066
Pimozide	10	0.108
Clofuprol	15	0.077
Benperidol	30	0.060
Bromoperidol	30	0.153
Spiroperidol	40	0.058
Penfluridol	60	0.466
Trifluoperidol	65	0.096
Haloperidol	75	0.152
Moperone	100	0.802
Droperidol	200	—
Fluanisone	500	3.44
Azaperone	600	—
Pipamperone	1,000	11.10
Phenothiazines and related agents		
Trifluoperazine	17	0.297
Fluphenazine	18	0.168
Triflupromazine	20	4.59
Chlorpromazine	21	12.00
Promazine	30	33.00
α-Flupenthixol	35	0.099
β-Flupenthixol	35	—
cis-Thiothixene	38	0.393
trans-Thiothixene	45	—

*Concentrations which inhibit 50%.

†For clinical potencies, the midpoint values of daily dose ranges²⁸⁻³¹ were mean and converted to μmol kg⁻¹ assuming a human weight of 70 kg.

Six concentrations of each drug were evaluated in triplicate to obtain IC₅₀ values. Data are the mean of three determinations which varied less than 15%.

binding, it is 160 times more potent than (–)-butaclamol in competing for ³H-dopamine binding. Similarly α-flupenthixol and *cis*-thiothixene are less than 1% as potent in inhibiting ³H-dopamine as ³H-haloperidol binding yet they still display stereospecificity with respect to their corresponding isomers. Interestingly flupenthixol and thiothixene are just as potent in competing for ³H-QNB as for ³H-dopamine binding, but they display no stereospecificity in inhibiting ³H-QNB binding.

Relative potencies of numerous neuroleptics as inhibitors of ³H-haloperidol binding to the dopamine receptor correlate well with clinical efficacy^{14,23,24}. There is, however, a much weaker correlation of neuroleptic clinical and pharmacological potency with ³H-dopamine binding, in spite of the fact that both dopamine and haloperidol presumably interact with the same receptor. The differences between interactions of ³H-haloperidol and ³H-dopamine with the dopamine receptor seem to stem from their labelling two different states of the dopamine receptor. Agonists are much more potent in competing for ³H-dopamine than ³H-haloperidol binding. Conversely, antagonists have substantially higher affinity for haloperidol than dopamine-binding sites. Thus dopamine and haloperidol respectively label discrete "agonist" and "antagonist" states of the dopamine receptor¹⁴. This two-state model for the dopamine receptor accords with data supporting such a model for the 5-HT, α-noradrenergic and opiate receptors²⁵. One would expect pharmacological activities of antagonists to be related better to their affinities for antagonist than agonist states of the receptor, which explains the better correlation of neuroleptic clinical activity with affinity for haloperidol than for dopamine binding²³.

The binding of ³H-LSD and ³H-5-HT is also inhibited stereospecifically and with considerable potency by these neuroleptics. The neuroleptics display about the same potency in inhibiting ³H-LSD as ³H-dopamine binding as well as a similar degree of stereospecificity. Thus (+)-butaclamol is 140 times as potent as (–)-butaclamol in lowering ³H-LSD binding and is about 160 times as potent as the (–)-isomer in lowering ³H-DHA binding. The neuroleptics are somewhat less

potent in lowering the binding of ³H-5-HT, but still display a degree of stereospecificity. The potencies of these neuroleptics in inhibiting ³H-dopamine and ³H-LSD binding are similar to their potencies as inhibitors of the dopamine-sensitive adenylate cyclase^{2,3}. In spite of these stereospecific effects, blockade of 5-HT receptors is probably not a major source of neuroleptic clinical efficacy, because relative influences of an extensive series of these drugs on ³H-LSD binding do not correlate with clinical potency¹⁷.

Since the chemical structure of butyrophenones resembles that of the inhibitory neurotransmitter GABA¹, it was also of interest to evaluate the influence of butyrophenones on GABA uptake by synaptosome-enriched brain homogenates. In this type of tissue preparation ³H-GABA is accumulated primarily by nerve terminals^{26,27}. The butyrophenones examined are all capable of inhibiting ³H-GABA uptake (Table 2). The most potent, fluspirilene, reduces uptake 50% at 3 μM, similar to its potency in inhibiting ³H-dopamine binding and in inhibiting the dopamine-sensitive adenylate cyclase^{2,3}. There is a several hundredfold variation in the relative potencies of the butyrophenones in reducing GABA uptake, and these variations correlate well with the relative clinical potencies of the drugs ($r = 0.86$, $P < 0.001$). If inhibition of GABA uptake were a common mechanism whereby therapeutic effects of all neuroleptics were elicited, then the phenothiazines and related agents should also show a similar correlation between inhibition of GABA uptake and clinical potency. We detect no correlation, however, between the influence of these agents on GABA uptake and their clinical effects. The very potent neuroleptic α-flupenthixol is no more effective in reducing GABA uptake than the weak neuroleptic promazine. Similarly, the potent neuroleptic fluphenazine has about the same potency as the moderately effective neuroleptic chlorpromazine.

The most striking finding indicating that the pharmacological activity of neuroleptics related to phenothiazine cannot be attributed to influences on GABA uptake derives from experiments with stereoisomers. No difference is apparent in the potencies of α- and β-flupenthixol in competing for GABA uptake in spite of the fact that essentially all the pharmacological activity resides in the α-isomer with β-flupenthixol being essentially inactive⁸. Similarly, the *cis*- and *trans*-isomers of thiothixene have the same affinities for GABA uptake sites, though only *cis*-thiothixene has clinical and pharmacological activity⁷.

It could be argued that the correlation between relative potencies of butyrophenones in inhibiting GABA uptake and their clinical potencies indicates that the therapeutic actions of butyrophenones may be mediated at least in part by inhibition of GABA uptake. Since uptake of GABA into nerve terminals is thought to inactivate synaptically released GABA, such an effect would potentiate the synaptic effects of GABA, which in turn could inhibit the firing of dopamine neurones. Similarly inhibition of opiate receptor binding by butyrophenones but not by phenothiazines correlates well with clinical antischizophrenic activity ($r = 0.78$, $P < 0.01$ with no added Na⁺; $r = 0.61$, $P < 0.05$ with 100 mM Na⁺)³². There is also some correlation between antipsychotic potencies of neuroleptics and inhibition of dopamine release⁵. Before the identification of ³H-haloperidol binding to dopamine receptor sites^{14,23,24} these formulations might have represented plausible mechanisms for clinical effects of butyrophenones if not of phenothiazines. But since the butyrophenones are about 100–1,000 times more potent in competing for ³H-haloperidol binding to dopamine receptor sites than in reducing GABA uptake, opiate binding or dopamine release and since inhibition of haloperidol binding predicts phenothiazine as well as butyrophenone activities, a more parsimonious explanation attributes clinical actions of both butyrophenones and phenothiazines to blockade of dopamine receptors^{14,23,24}.

The correlation between the influences of these drugs on ³H-GABA uptake and clinical potencies emphasises the difficulties inherent in evaluating correlations between bio-

chemical and clinical effects of drugs. Similarly, one should be circumspect about concluding that stereospecificity of biochemical effects "proves" that one is dealing with the pharmacologically relevant site of a drug's action. Thus, if a given behavioural effect in animals is elicited more by (+)-butaclamol than by (–)-butaclamol, it should not automatically be assumed that the behaviour is dopamine mediated, since the drug effect might also involve 5-HT receptors.

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Transmitter metabolism in substantia nigra after inhibition of dopaminergic neurones by butyrolactone

DOPAMINERGIC nigrostriatal neurones have recently been found to contain dopamine (DA) also in their dendrites¹. Certain observations suggest a release of transmitter from these dendrites: depolarisation by potassium ions induces a release of ³H-DA from incubated tissue slices of rat substantia nigra², and stimulation of the median forebrain bundle increases 3,4-dihydroxyphenylacetic acid, a major metabolic of DA³, in the mesencephalic region containing dopaminergic cell bodies and dendrites⁴, as well as in caudate-putamen, the terminal area. These similarities

between terminal and somato-dendritic areas point to a similar regulation of DA metabolism and an active role of DA in both parts of the neurone. We have studied this question in a situation where the dopaminergic neurones are inhibited. γ -Butyrolactone (GBL) applied systemically, strongly reduces unit activity of the dopaminergic neurones in zona compacta of substantia nigra⁵. Under these conditions, the changes observed in the somato-dendritic complex differed markedly from those reported for the terminal area^{6–12}.

In male rats (200–300 g) of a random-bred SIV (Sprague-Dawley-Ivanovas) strain, the somato-dendritic complex was analysed as a whole with biochemical techniques, while DA nerve cell bodies were studied by histochemical microfluorimetry¹³. For biochemical analyses the brains were quickly removed after decapitation, placed on dry ice and covered with carbon dioxide snow. Standardised tissue cylinders (about 1 mg) from substantia nigra were obtained as described in Fig. 1¹⁴. These cylinders contained the medial two thirds of zona compacta and small amounts of tissue from zona reticulata and lemniscus medialis. Their localisation corresponds to the area of highest dendritic density¹ and to the highest DA concentration in substantia nigra (F. Hefti and W. Lichtensteiger, unpublished). DA concentration was determined in individual tissue cylinders with an enzymatic-isotopic method¹⁵. The rate of DA synthesis was assessed by measuring accumulation of dopa after inhibition of dopa-decarboxylase with 3-hydroxybenzylhydrazine (NSD 1015, Sandev) with a newly developed enzymatic-isotopic assay¹⁶. In these conditions dopa accumulated linearly in substantia nigra cylinders up to 60 min (ref. 16).

Both inhibition of dopaminergic cells by GBL^{5,9} and interruption of impulse flow by axotomy^{9,10,12} have been found to lead to an increase in the concentration of dopamine in caudate-putamen. After a single injection of 750 mg kg⁻¹ of GBL, DA concentration was greatly increased between 15 and 150 min (ref. 8). We could confirm

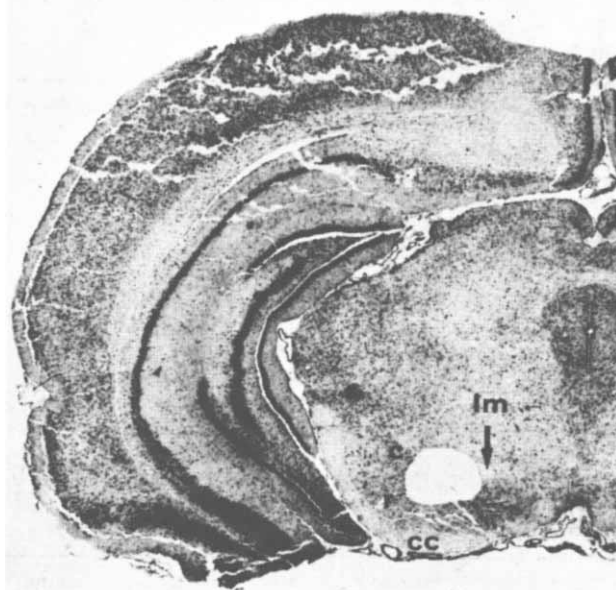


Fig. 1 Localisation of standardised tissue cylinders in substantia nigra. From frozen brain, a 1-mm thick frontal slice was sectioned anteroposteriorly from level A 1400 to level A 2400 according to the atlas of Koenig and Klippel²⁰. A cylinder of 1-mm diameter was punched out on each side. The figure shows the histological control of such a slice fixed after punching (Toluidine blue staining). c, Zona compacta; r, zona reticulata of substantia nigra; Im, lemniscus medialis; cc, crus cerebri.

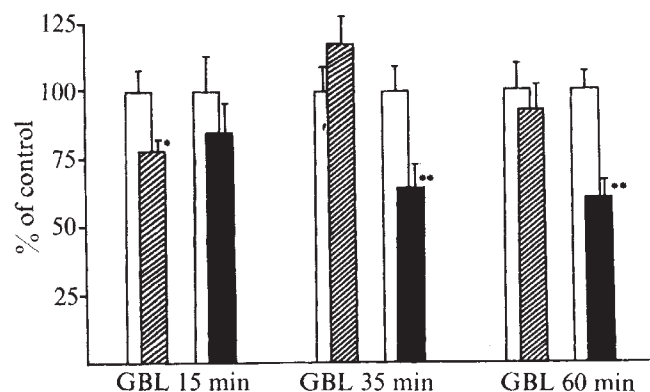


Fig. 2 Effect of γ -butyrolactone (GBL) on DA concentration (hatched bars) and dopa accumulated in substantia nigra 30 min after injection of the dopa-decarboxylase inhibitor NSD 1015 (100 mg kg⁻¹ intraperitoneally; solid bars). Open bars represent saline injected controls. (Mean $\pm$ s.e.m.; $n = 9-10$; * $P < 0.025$; ** $P < 0.005$). Concentration of DA and dopa were based on protein content of tissue cylinders²¹. Mean $\pm$ s.e.m. of DA control levels was 15.0 ± 1.1 ng per mg protein, mean of dopa accumulated in 30 min was 8.3 ± 0.8 ng per mg protein in control animals.

this effect in rats (at 60 min) and observed a comparable change in mice (at 10 and 90 min; unpublished). In contrast to this the DA concentration in substantia nigra was not raised after 35 and 60 min; there was even a reduction after 15 min (Fig. 2). The quantitative histochemical investigation of DA perikarya in zona compacta of substantia nigra revealed no changes in fluorescence intensity in male rats 60 min after GBL, while intensity tended to decrease in male mice at 10 min and 90 min after drug administration (Table 1). This is again at variance with the response of DA terminals, where GBL elicits a marked rise in fluorescence intensity^{7,17}.

Also with regard to DA synthesis, our observations on the somato-dendritic area (Fig. 2) contrast with the findings in the terminal region^{10,11}. In substantia nigra, dopa accumulation remained about 40% below control level at

35 and 60 min, whereas the amount of dopa accumulated in caudate-putamen has been shown to be more than doubled 35 min after GBL and to be again at control levels after 90 min¹⁰.

Since the decarboxylase inhibitor NSD1015 was reported to slightly inhibit monoamine oxidase¹⁸, it could be argued that the absence of an increase in DA synthesis rate in substantia nigra might be related to effects on intraneuronal feedback mechanisms on tyrosine hydroxylase. The effects of GBL on dopa accumulation in caudate-putamen were studied with another inhibitor of dopa-decarboxylase, *N*¹-(DL-seryl)-*N*²-(2,3,4-trihydroxybenzyl)-hydrazine (Ro 4-4602) (ref. 10). In caudate-putamen, however, an increased accumulation of dopa was found after inhibition of impulse flow by axotomy also with NSD1015 (ref. 11). Moreover, Walters and Roth¹⁹ have shown that prior application of an inhibitor of monoamine oxidase does not prevent the increase in dopa accumulation in caudate-putamen after Ro 4-4602 and GBL, only the magnitude of the effect was slightly reduced. Inhibition of monoamine oxidase for 30 min did not derude dopa accumulation after Ro 4-4602 alone. Therefore, it seems that the discrepancies between substantia nigra and caudate-putamen are not related to the type of decarboxylase inhibitor used, but rather reflect a true difference between the two regions.

Our findings indicate that somatodendritic and terminal areas of dopaminergic neurones differ with regard to the regulation of monoamine metabolism at least in some functional states. The relative importance of biochemical changes in dendrites and perikarya is as yet difficult to assess. A rise in dendritic DA after GBL may have been obscured by a fall in DA concentration of perikarya, or else, it may have been too small to be detected against the background of perikaryal DA. Our microfluorimetric data speak against the first possibility. Moreover, the contribution of dendrites to nigral DA seems to be considerable, as DA concentration in zona reticulata, which contains mainly dendrites, is relatively high (4.2 ± 0.7 ng per mg protein; mean $\pm$ s.e.m.; $n = 10$) as compared to zona compacta containing a mixture of perikarya and dendrites (14.9 ± 1.5 ng per mg protein; $n = 10$). It follows from these considerations that the dopaminergic dendrites themselves probably responded to GBL in a way different from the reaction of terminals.

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Table 1 Fluorescence intensity of dopamine-containing nerve cells in zona compacta of substantia nigra*

Treatment	Mean relative fluorescence intensity†	Mean $\pm$ s.d. of logarithmically transformed intensity distribution (natural log)	Cell count (No. of animals)
Male rats			
Saline, 60 min	60.58	4.044 ± 0.3602	779 (5)
GBL 750 mg kg ⁻¹ , 60 min	62.44	4.063 ± 0.3842	762 (5)
Male mice			
Saline, 10 min	51.32	3.861 ± 0.4150	749 (5)
Saline, 40 min	52.47	3.866 ± 0.4548	803 (5)
GBL 375 mg kg ⁻¹ , 10 min	48.42	3.790 ± 0.4515 ‡	782 (5)
GBL 375 mg kg ⁻¹ , 90 min	48.11	3.755 ± 0.5382 ‡	781 (5)

*The nigral DA neurone group was investigated in 8 frontal 7- μ m sections (distance between sections = 35 μ m). Twenty neighbouring cells were measured in each section, starting at the dorsolateral corner of zona compacta (for further details, compare refs 13 and 22). The experiments on mice were performed in the course of a study on morphine action; controls are taken from that study²².

†Mean relative intensity in per cent of noradrenaline standard. For statistical analysis, the values of individual cells were logarithmically transformed. Mean values were compared according to Scheffé's method of linear contrasts¹³.

‡Different from both saline controls for $P < 0.05$.

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Technique for studying synaptic connections of single motoneurons in man

DURING normal voluntary muscle contractions, the pattern of discharge of a single motoneurone is determined by the summed effects of an enormous variety of different types of synaptic input. In principle, the effect of any one of these inputs could be determined by constructing a histogram of the time of occurrence of motoneurone spikes after repeated presentation of a suitably controlled stimulus^{1,2}. This procedure extracts from the naturally occurring spike train only those changes in firing time locked to the stimulus. The synaptic effect of a given input is thus revealed in terms of the specific contribution it makes to the total firing pattern of the cell. Such studies are

maximum at 76 ms. This is followed by some rather slow fluctuations superimposed on a prolonged period of reduced probability which eventually returns to control about 300 ms after the initial stimulus⁵. The timing of these responses suggests that the earliest excitation and inhibition involve spinal pathways and that the later more pronounced excitation involves supraspinal pathways^{6,7}.

The possibility that these responses might be due to vibration transmitted to muscle receptors has been tested by anaesthetising the index finger. The responses can be abolished either by a 2% Lignocaine or by an ischaemic block of the digital nerves. Moreover, weak electrical stimulation of the digital nerves at two times threshold for perception gives essentially the same result as tapping the nail.

The scale of the response to such a modest and limited stimulus, illustrates the most powerful influence that a cutaneous afferent volley can exert on the discharge of a motoneurone. During the initial inhibitory phase, the unit is almost completely silenced. Immediately after this, the mean firing rate is more than doubled. It is clear that the results of experiments in which the response of the motor system is studied after sudden external disturbance must be interpreted with caution if any sudden displacements of the skin are involved⁸.

The results of the present type of experiment gain additional

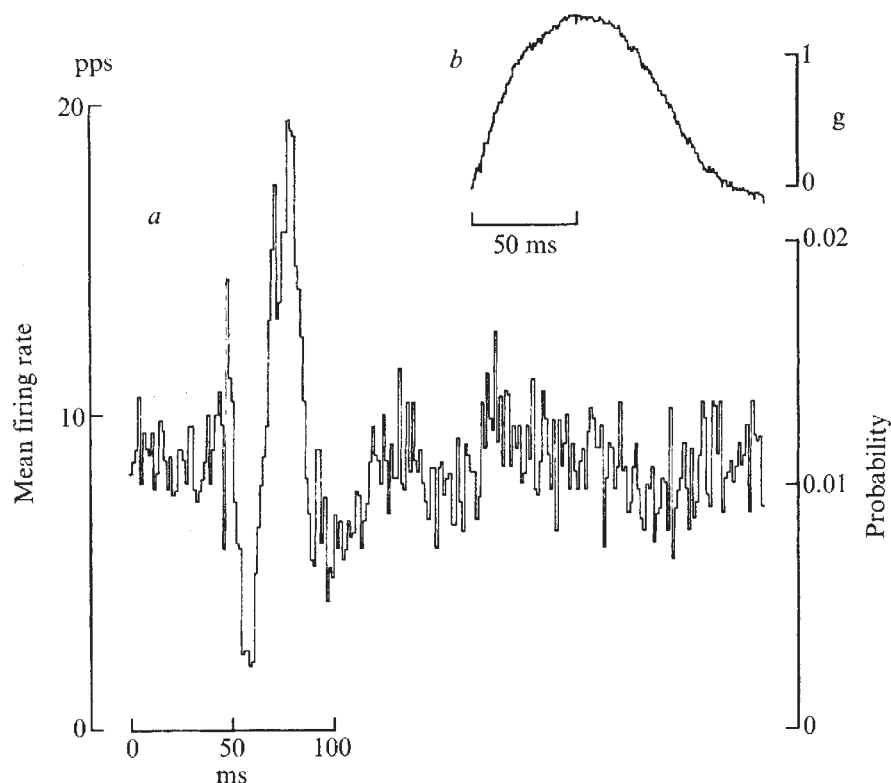


Fig. 1 *a*, Post-stimulus time histogram of the occurrence of the action potential of a single motor unit in the first dorsal interosseous muscle after a sharp tap to the tip of the nail of the index finger. Stimuli delivered at 3 s^{-1} while the subject maintained an isometric contraction such that the unit fired steadily at about 8 p.p.s. 4,096 sweeps. Bin width 1.28 ms. Stimulus given at time zero. *b*, Twitch response of the same motor unit recorded by averaging the force of abduction of the index finger exerted at the second proximal interphalangeal joint triggering the averager from the motor unit action potential while the subject maintained a contraction such that the unit fired at ≤ 3 p.p.s. For details of method see refs 3 and 4. 64 sweeps. Threshold of recruitment 160 g.

most conveniently carried out in man where the cooperation of the subject is invaluable. This report describes an example of this approach in the study of the synaptic effects of a simple cutaneous input. In more general terms, the experimental situation described enables, for the first time, the synaptic connections of single motoneurons to be examined in man, studies previously only attempted in anaesthetised or decerebrate animals.

Figure 1*a* shows the changes in probability of firing of a single motor unit in a hand muscle after a sharp mechanical tap to the tip of a fingernail. At 39 ms after the tap there is an increase in probability of occurrence of the motor unit action potential which reaches a maximum 7 ms later. This period of excitation is immediately followed by a period of inhibition during which the probability of motor unit firing reaches a minimum at 58 ms. This itself is terminated by another more pronounced and prolonged period of excitation reaching a

significance because the reflex connections of a motoneurone can be related to its recruitment threshold during voluntary muscle contraction and to the mechanical properties of the muscle fibres it innervates (Fig. 1*b*). It will now be possible, in man, to test the applicability of the unexpected subtleties revealed by intracellular recording in the synaptic connections of cat hind limb motor units with different mechanical properties^{9,10}.

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Voltage-dependent action of tetrodotoxin in mammalian cardiac muscle

TETRODOTOXIN (TTX) in many excitable membranes selectively blocks the transient change in sodium conductance which underlies the rising phase of the action potential. Electrophysiological and binding studies from nerve and skeletal muscle^{1–5} indicate that a one-to-one binding reaction between toxin and Na channels with a dissociation constant of about 5×10^{-9} M can adequately describe the kinetic and steady-state actions of TTX on the Na conductance. Cardiac muscle is unusual in that it shows a marked insensitivity to TTX. A substantial reduction in the rate of rise of action potentials in ventricular trabeculae and Purkinje fibres does not occur until a TTX concentration of about 10^{-6} M is reached^{6–8}. We report here the results of a systematic study of the effects of TTX on mammalian cardiac muscle. The experiments were designed to determine whether the reported difference in sensitivity to TTX is an indication of basic differences in the TTX receptor of cardiac muscle as compared to nerve and skeletal muscle. Unlike any TTX effects reported in other tissues⁸, our results show a voltage dependence of TTX block. This suggests possible differences in the structure of the TTX binding site or of the Na channel itself.

Intracellular membrane action potentials were recorded from guinea pig papillary muscles by conventional microelectrode techniques, and the maximal upstroke velocities ($\dot{V}_{\max}$) of the action potentials were differentiated electronically. The papillary muscles were mounted in a single sucrose gap arrangement as described previously⁹. The sucrose gap permitted uniform displacement of the membrane potential in the short (<0.8 mm) muscle end where action potentials were recorded. The muscles were stimulated with brief current pulses (5 ms; 0.3 Hz), and

occasionally long lasting depolarising or hyperpolarising constant currents were applied. Our experiments showed that $\dot{V}_{\max}$ was linearly related to the extracellular Na concentration (correlation coefficient, $r = 0.99$), indicating that the corresponding membrane current is carried by Na ions as in other excitable tissues. The blocking action of TTX on $\dot{V}_{\max}$ reached a steady state in about 5 min, and no further effect of the toxin was seen after continuous exposure for up to 2 h. For concentration–response curves, $\dot{V}_{\max}$ measurements were typically made after 15 min equilibration in solutions containing toxin and can be considered as steady state. Continuous microelectrode impalements in a single cell were routinely maintained throughout an experiment allowing a direct comparison of the effects of several toxin concentrations on a given cell at two membrane potentials. Membrane resting potential, V_r , was either changed by increasing the potassium concentration in normal Tyrode's solution (5.4 K⁺-Tyrode's) to 10.8 mM (10.8 K⁺-Tyrode's) or by passing constant currents across the sucrose gap.

The concentration dependence of TTX inhibition of $\dot{V}_{\max}$ is shown in Fig. 1. The curves were fitted by assuming a first-order reaction between toxin and receptor. The half-maximal inhibition of $\dot{V}_{\max}$ in 5.4 K⁺-Tyrode's occurs at 1.4×10^{-5} M TTX. Doubling the potassium concentration of the Tyrode's solution profoundly affects the sensitivity of the muscle to TTX. Only 1.2×10^{-6} M TTX is required for half-maximal inhibition of $\dot{V}_{\max}$ in 10.8 K⁺-Tyrode's. The high potassium solution depolarised the fibres from -89 ± 3 mV to -71 ± 3 mV ($\pm$ s.e.). The increase in apparent affinity to TTX is a result of this depolarisation and not of the increased K⁺ concentration *per se*, since a depolarisation of similar magnitude produced by passing a constant current through the fibre had a similar effect on the TTX sensitivity.

$\dot{V}_{\max}$ is an indirect and nonlinear measure of the maximal sodium conductance g_{Na} (refs 3 and 10) which is actually the parameter directly affected by TTX binding^{1,3,6}. We thought it possible that the depolarisation induced decrease in the control value of $\dot{V}_{\max}$ (Figs 1 and 2) might change the relationship between $\dot{V}_{\max}$ and g_{Na} (ref 10), which in turn could alter the apparent sensitivity to TTX. Therefore, we carried out experiments with 5.4 K⁺-Tyrode's solution in which 50% of the Na⁺ had been replaced with choline⁺. In these conditions $\dot{V}_{\max}$ is reduced by half—that is, even more than in 10.8 K⁺-Tyrode's. There was a small, non-significant ($P > 0.1$) difference in the concentration–response curves in normal and low Na⁺-Tyrode's which could not explain the depolarisation-induced

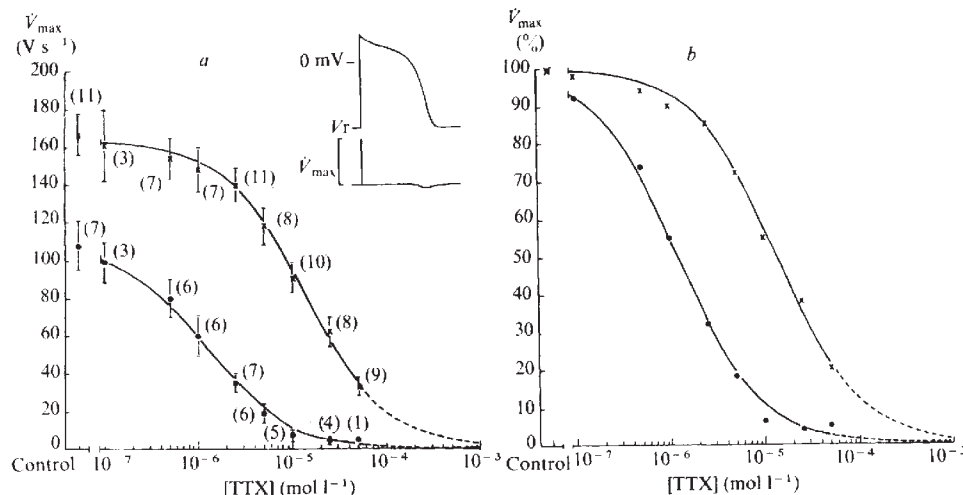


Fig. 1 *a*, Concentration–response curves showing the effects of TTX (log scale) on maximum upstroke velocity ($\dot{V}_{\max}$; mean $\pm$ s.e.) of the action potential in 5.4 (x) and 10.8 (●) mM K⁺-Tyrode's. The inset is a schematic drawing of resting potential (V_r) and action potential (upper trace); the latter was differentiated electronically (lower trace) and the maximal upstroke velocity ($\dot{V}_{\max}$) was measured. *b*, Normalised mean values of *a*. Solid lines are concentration–response curves calculated from the

equation

$$\dot{V}_{\max \text{ TTX}} = \dot{V}_{\max \text{ control}} (1 - [1/(1 + \{K_m/[TTX]\})])$$

$\dot{V}_{\max \text{ control}}$ and K_m were 167 V s⁻¹ and 1.4×10^{-5} mol l⁻¹ in 5.4 K⁺-Tyrode's, and 109 V s⁻¹ and 1.2×10^{-6} mol l⁻¹ in 10.8 K⁺-Tyrode's. Numbers of determinations per mean value given in parentheses in *a*.

increase in sensitivity to TTX. We do not yet know whether the measurements of $\dot{V}_{\max}$ reflect directly the true binding behaviour of TTX to the Na channels. Studies on skeletal muscle, however, show that measurements of $\dot{V}_{\max}$ give values for the apparent dissociation constant of TTX which agree within factors of 2–6 with those obtained from binding studies^{4,5,11}.

The concentration–response curves in Fig. 2 were taken from a single papillary muscle which was treated with various TTX concentrations in 10.8 and 5.4 K⁺-Tyrode's. Initially the preparation was superfused with 10.8 K⁺-Tyrode's and the concentration of TTX was increased until $\dot{V}_{\max}$ was completely

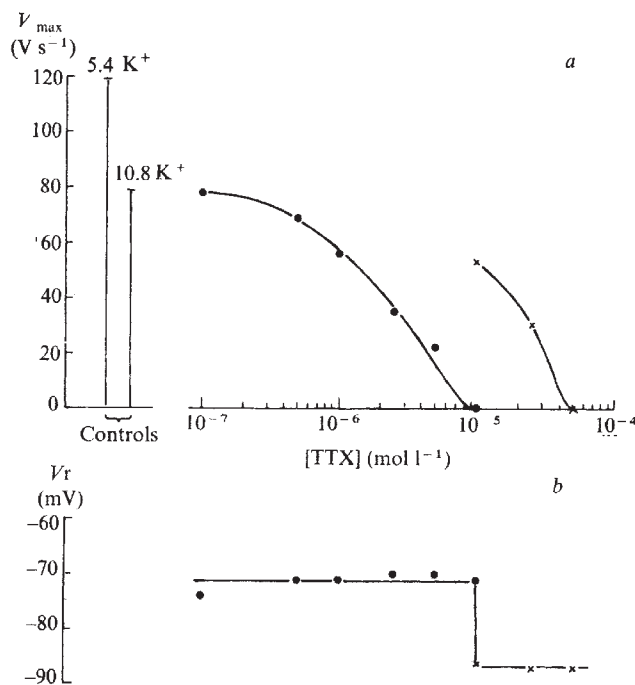


Fig. 2 Concentration–response curves of TTX on $\dot{V}_{\max}$ in 10.8 (●) and 5.4 (×) mM K⁺-Tyrode's. Data were collected from one papillary muscle during a single microelectrode impalement. *a*, Bars on the left give control values of $\dot{V}_{\max}$ before application of TTX. Initially, the preparation was bathed in 10.8 K⁺-Tyrode's and the TTX concentration (log scale) increased until $\dot{V}_{\max}$ fell to zero; 5.4 K⁺-Tyrode's containing 10⁻⁵ mol l⁻¹ TTX was then applied and $\dot{V}_{\max}$ returned to 45% of its normal value and fell again to zero upon further increase in the TTX concentration. *b*, Changes in the resting potential (V_r) caused by the variation in potassium concentration.

abolished, even after increasing the stimulus strength. When the solution was changed to 5.4 K⁺-Tyrode's containing the same amount of toxin that had caused a complete block of $\dot{V}_{\max}$ in 10.8 K⁺-Tyrode's, a rapid recovery of $\dot{V}_{\max}$ to about 50% of its control value occurred, while V_r hyperpolarised by 16 mV. When the TTX concentration was increased further, the fibre again showed the lower sensitivity to toxin expected at this V_r . One possible explanation of this effect is that the Na channels inactivated during depolarisation are protected from TTX binding, but become sensitive to TTX again on hyperpolarisation when inactivation is removed.

We have tested this possibility by incubating non-stimulated muscles for 10 min with TTX (10⁻⁵ M) at -85 mV where all Na channels are available and at -50 mV (20 K⁺-Tyrode's) where they are fully inactivated^{9,12}. When the muscles were rapidly hyperpolarised by passing a constant current and stimulated from a V_r of about -90 mV, the degree of inhibition of $\dot{V}_{\max}$ was slightly larger following the period of inactivation. This suggests that the depolarisation induced inactivation of the Na channels did not protect them from the TTX action. Therefore, we explain the result in Fig. 2 in terms of a release

of TTX during hyperpolarisation to -87 mV from Na channels which were blocked by the toxin at -71 mV. In other words, the hyperpolarisation partially removed the TTX block attained at the lower V_r . This argues strongly for voltage dependence of TTX binding.

Our results indicate at least two major differences between the action of TTX in cardiac muscle and in nerve or skeletal muscle. First, $\dot{V}_{\max}$ of action potentials of cardiac muscle is three to four orders of magnitude less sensitive to the toxin. Second, the sensitivity of cardiac fibres to TTX is voltage dependent, a depolarisation of approximately 18 mV from the normal V_r being sufficient to change the apparent affinity by a factor of 10. The voltage-dependent effects of TTX on cardiac muscle indicate to us a basic difference in the chemistry or configuration of the TTX receptor, or of the Na channel¹³. Possible differences in the structure of the Na channel in cardiac muscle as compared with other excitable tissues are relevant to the mechanism of action of antiarrhythmic drugs, which primarily affect the Na conductance system.

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Localisation of gonadotropin-releasing and thyrotropin-releasing hormones in human brain by radioimmunoassay

GONADOTROPIN-RELEASING hormone (GnRH) and thyrotropin-releasing hormone (TRH) are two hypothalamic oligopeptides which regulate the secretion of gonadotropins and of thyroid-stimulating hormone (TSH), respectively, from the anterior pituitary^{1,2}. It is generally accepted that these neurohormones are produced by nerve cells, transmitted along axons and stored in nerve endings; when released, they are transferred by way of the hypothalamo–hypophyseal portal system to the anterior pituitary³. Human brains of adults have been shown to contain GnRH and TRH activity⁴ but quantitative measurements of these hormones are available for brains of aborted foetuses only⁵. This report deals with the distribution of GnRH and TRH in the adult human brain, their possible sites of formation, routes of migration and sites of storage in the pituitary stalk.

The hypothalami and other brain regions were removed during routine autopsies carried out at the Institute of Forensic Medicine, Tel Aviv. The tissues were obtained from three 22–40-yr-old females and twelve 41–95-yr-old males, most of them killed in road accidents. The bodies were kept for 6–48 h at 4 °C until autopsy. The pituitary stalk and gland were dissected

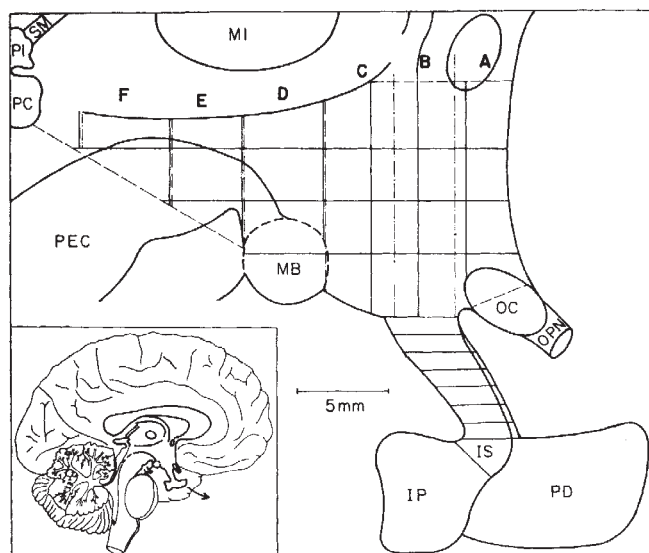


Fig. 1 Outline of the dissection of the human hypothalamus and pituitary stalk. Hypothalamus was dissected in the coronal planes to six strips (broken lines, A-F) or into 25 blocks (solid lines). The pituitary stalk was sectioned into eight portions (including the infundibular stem). Insert: mid-sagittal section of the human brain. AC, Anterior commissure; CF, column of fornix; DM, dorsomedial nucleus; HS, hypothalamic sulcus; IN, infundibular nucleus; INF, infundibulum of neurohypophysis; IP, infundibular process of neurohypophysis; IS, infundibular stem of neurohypophysis; MB, mammillary body; MI, massa intermedia; OC, optic chiasma; ON, oculomotor nerve; OPN, optic nerve; PC, posterior commissure; PD, pars distalis of adenohypophysis; PEC, peduncle of cerebrum; PI, pineal body; PO, preoptic nucleus; PON, pons; PN, posterior nucleus; PT, pars tuberalis of adenohypophysis; PV, paraventricular nucleus; SO, supraoptic nucleus; SM, stria medullaris; VM, ventromedial nucleus.

to 48 h after death. Male rats (12 per group) were killed with ether. In one group, the hypothalami were removed and extracted immediately, and the other group kept for 48 h at 4 °C before removal and extraction of hypothalami. Hypothalamic content of GnRH was 4.4 ± 0.3 ng at death compared with 4.0 ± 0.3 ng 48 h later, and of TRH was 7.4 ± 0.1 compared with 7.2 ± 0.3 ng.

The content of GnRH and of TRH in the human hypothalamus were initially estimated by assaying the dissected strips (Fig. 1 A-F). The results are summarised in Table 1. The concentration of GnRH was the highest in the rostral strip

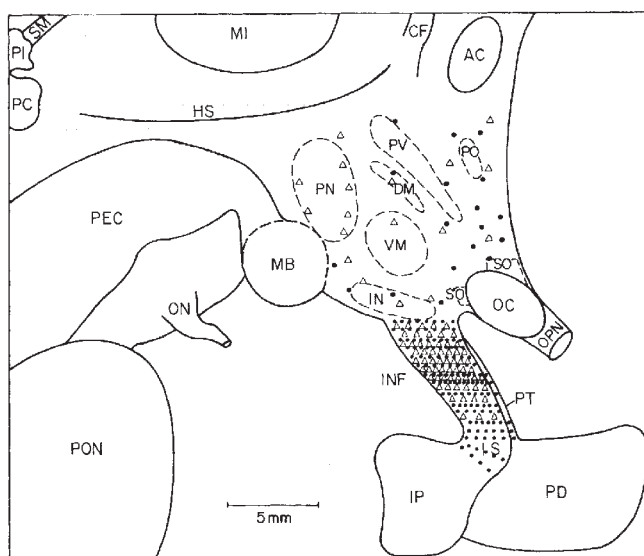


Fig. 2 Localisation of GnRH (●) and of TRH (Δ) in the human hypothalamus and pituitary stalk. The outline of hypothalamic nuclei in the sagittal plane are shown in broken lines. Each dot or triangle represents a concentration of 70–110 pg/mg tissue of the corresponding neurohormone. For abbreviations, see Fig. 1.

from the hypothalamus. Each brain was cut midsagittally and each half of the hypothalamus was sectioned in the coronal plane to six strips (Fig. 1 A-F). In four brains one half of the hypothalamus was sectioned into 25 blocks (Fig. 1); the pituitary stalk was sectioned into seven horizontal slices and the infundibular stem, infundibular process (posterior pituitary) and the pars distalis (adenohypophysis) were also collected. Each of the sectioned pieces was weighed, extracted and analysed for GnRH and TRH by radioimmunoassay as described previously^{6,7}.

In a preliminary study we have found that in the rat the hypothalamic content of GnRH and TRH did not change up

(A), which comprises the preoptic area, and declined progressively towards the caudal strip (F), which contained negligible amounts of GnRH. A different pattern of distribution was observed for TRH (Table 1): the highest concentration was found in strip C which consists of the posterior nucleus and the premammillary area. Radioimmunoassay of extracts derived from hypothalamic tissue blocks (Fig. 1), enabled us to follow more precisely the localisation of GnRH and TRH. GnRH was localised mainly in the preoptic area (Fig. 2), between the

Table 1 GnRH and TRH concentrations in the human brain

Sample	Weight (mg)	GnRH (pg per mg tissue)	TRH (pg per mg tissue)	Total GnRH (ng)	Total TRH (ng)
Hypothalamus					
Strip A	72 ± 14	130 ± 59	54 ± 10	9.36	3.88
B	98 ± 16	62 ± 24	73 ± 8	6.07	7.15
C	96 ± 5	27 ± 8.3	101 ± 14	2.59	9.69
D	105 ± 16	11 ± 1.6	69 ± 10	1.15	7.24
E	68 ± 5	12 ± 0.6	38 ± 12	0.81	2.58
F	54 ± 8	5.4 ± 3	5.2 ± 3	0.29	0.28
Pituitary stalk*	41 ± 3	1,430 ± 334	855 ± 145	20.27	30.82
Infundibular process	82 ± 3	< 2	< 0.5	58.63	35.05
Pars distalis	328 ± 48	< 2	< 0.5		
Pineal body	95 ± 20	< 2	1.1 ± 0.8		
Thalamus	91 ± 12	< 2	9.2 ± 2.6		
Cerebral cortex	100 ± 11	< 2	1.0 ± 0.4		

*Including infundibular stem.

Each tissue sample was weighed, extracted and analysed by radioimmunoassay for GnRH and TRH content. Mean ± s.e.m. for 10–15 samples, are shown. Total amount of the hormones in hypothalamic strips (A–F) represents the content of half a hypothalamus, dissected in the midsagittal plane.

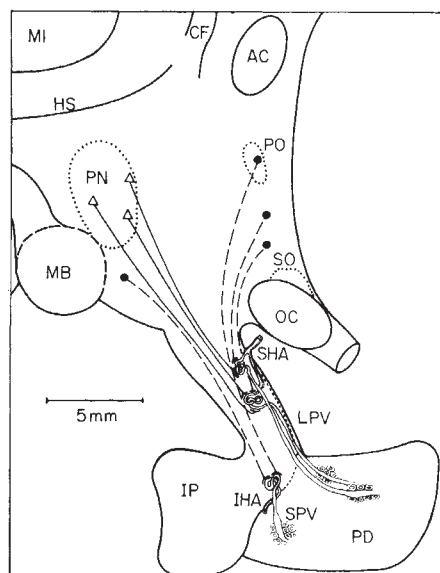


Fig. 3 Scheme of the neurovascular pathway of GnRH and TRH in the human brain. ●, Cell bodies of TRH-producing neurones in the posterior nucleus (PN). TRH is transferred by axons (solid lines) to the capillary loops in the pituitary stalk and reach the pars distalis (PD) of the adenohypophysis by way of the long portal vessels (LPV). ●, Cell bodies of GnRH-producing neurones in the preoptic area of the hypothalamus. Their axons (broken lines) terminate on the capillary loops of the superior hypophyseal artery (SHA) and the inferior hypophyseal artery (IHA), and reach the adenohypophysis by way of both the long (LPV) and short (SPV) portal vessels. The location of the portal vessels was drawn according to Daniel and Prichard⁸. For abbreviations, see Fig. 1.

supraoptic and the preoptic nuclei (340 pg per mg tissue); high concentration was also found in an area located anterior to the mammillary body in the basal hypothalamic area (186 pg per mg tissue). The highest concentration of TRH was observed in the posterior nucleus (158 pg per mg tissue) and in the region connecting this nucleus with the pituitary stalk. Exceedingly high concentrations of both neurohormones were present in the pituitary stalk (1,430 pg per mg tissue and 855 pg per mg tissue for GnRH and TRH, respectively; Table 1), with a difference in distribution (Fig. 2): GnRH was evenly distributed all along the pituitary stalk, whereas TRH was localised in the upper two-thirds (except for one case). The total content of GnRH in the hypothalamus was 40.5 ng and in the pituitary stalk 58.6 ng. Hypothalamic content of TRH was 61.7 ng and of the pituitary stalk 35 ng. No significant amounts of the hormones were detected in the different regions of the pituitary gland. Low concentrations of TRH were found in some extra-hypothalamic regions of the brain (Table 1). No apparent differences in the distribution or content of the two neurohormones were observed in relation to age or sex.

Axons descending from the hypothalamus have been shown to constitute the major component of the pituitary stalk⁸. Therefore, the extremely high concentrations of GnRH and TRH in the pituitary stalk (Fig. 2), may reflect the presence of pools of these hormones stored in axons and nerve terminals. Localisation of TRH and GnRH in specific hypothalamic regions may represent sites of synthesis of the hormones in neuronal cell bodies of these areas. In the rat, relatively high concentrations of GnRH have been found in the preoptic area and in the arcuate–median eminence region⁹. The median eminence of the rat, however, is anatomically homologous to the proximal portion of the human pituitary stalk⁸; therefore, the distributions of GnRH in human (that is, high concentrations in the preoptic area and the pituitary stalk) and rat hypothalamus, are similar.

An additional pathway—that is, by way of the cerebrospinal

fluid—has been suggested¹⁰ for the transfer of the neurohormones from their site of synthesis to their site of release. The high concentrations of GnRH in the lowest portion of the infundibular stem (Fig. 2), which is remote from the infundibular recess of the third ventricle, favours the direct neural route.

The absence of TRH in the lower third of the pituitary stalk implies that this neurohormone reaches the anterior pituitary by way of the long portal vessels (Fig. 3), whereas GnRH, which is evenly distributed along the whole pituitary stalk and infundibular stem, is transported by way of both the long and short portal vessels.

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Evidence for an extranucleolar mechanism of actinomycin D action

SINCE the original observation of Perry¹ that a low concentration of actinomycin D ($0.04 \mu\text{g ml}^{-1}$) selectively inhibits ribosomal RNA (rRNA) synthesis *in vivo*, this concentration of drug has been used by numerous investigators to selectively inhibit the synthesis of rRNA in cells in culture. Since nucleolar ribosomal DNA contains a high amount of deoxyguanosine and deoxycytosine bases² and actinomycin D selectively binds to deoxyguanosine³, it has been thought that actinomycin D preferentially binds to nucleolar DNA to inhibit the transcription of 45S ribosomal RNA precursor.

α -Amanitin, the peptide toxin from *Amanita phalloides*, also has inhibitory effects on the synthesis of rRNA^{4–6} and is known to produce nucleolar fragmentation^{7–10}. Since α -amanitin is a potent inhibitor of nucleoplasmic RNA polymerase II (refs 11–13) and protein synthesis is an integral component of rRNA synthesis^{14–17}, it is conceivable that α -amanitin and actinomycin D have comparable sites of action in inhibiting the transcription of mRNA(s) coding for the essential protein(s) required for the initiation of rRNA transcription *in vivo*. Although these observations with α -amanitin have suggested the possibility of an extranucleolar control of rRNA synthesis, a specific role of mRNA in this process remains to be demonstrated. In this report, evidence is presented for an extranucleolar mechanism of actinomycin D action suggesting that rRNA transcription in nucleoli is controlled by mRNA synthesis in the nucleoplasm.

Studies in this report were designed to ask two questions: (1) Is α -amanitin resistant transcription in isolated nuclei (RNA polymerase I + III) and purified nucleoli (RNA polymerase I) inhibited by the low concentrations of actinomycin D known to be effective *in vivo*; and/or, (2)

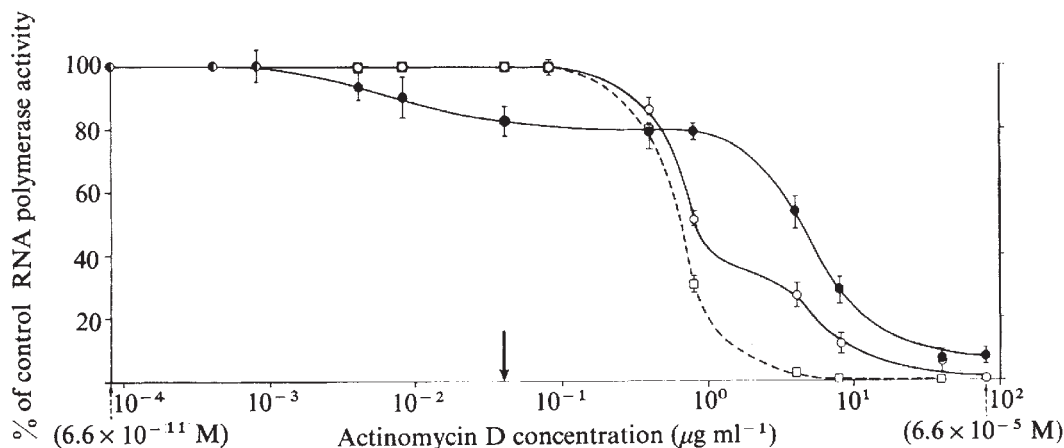


Fig. 1 Effect of actinomycin D on transcription in isolated nuclei and nucleoli. RNA polymerase assays, in the presence of varying concentrations of actinomycin D were performed as described below. Liver nuclei were isolated from adult male rats (300 g) by the method of Blobel and Potter¹⁸ with 1 mM phenylmethylsulphonylfluoride (PMSF) in all buffers according to Lindell¹⁹. Nucleoli were isolated from freshly prepared nuclei by resuspension of the nuclear pellet obtained above in 0.34 M sucrose. The nuclear suspension was then sonicated and fractionated according to Roeder and Rutter²⁰. The yield of nucleoli was 30–40% as calculated from the assay of RNA polymerase I activity in protein solubilised from starting nuclei and nucleolar product²¹. Routine preparations of nucleoli contain 0.5% RNA polymerase II by assay of RNA polymerase activity without exogenous DNA, plus and minus α -amanitin. RNA polymerase assays in rat liver nuclei and nucleoli were performed as described by Zerwekh *et al.*²² using a single concentration of $(\text{NH}_4)_2\text{SO}_4$ (0.05 M). Actinomycin D (Merck) was added in 10- μ l aliquots to 50- μ l nuclear samples to give final concentrations (in 125 μ l) ranging from 0.001 to 100 $\mu\text{g ml}^{-1}$ (6.6×10^{-11} – 6.6×10^{-5} M). Nuclei and nucleoli were preincubated with actinomycin D at room temperature for 10 min before initiation of the assays by addition of substrates and 5-³H-UTP (New England Nuclear, 25 Ci mmol⁻¹). Individual nuclear assays (5 in the absence and 5 in the presence of α -amanitin, 0.1 $\mu\text{g per assay}$) were incubated for 5 min at 30 °C. Incorporation of ³H-UMP was linear within this time period. Nucleolar assays were performed in the presence of α -amanitin to inhibit any possible residual RNA polymerase II activity. All incubations were terminated by pipetting 100- μ l aliquots of each assay on to Whatman DE-81 filter disks and immediately placing them in 5% Na_2HPO_4 . The filters were then washed, and counted as described by Lindell *et al.*¹¹. Nucleoplasmic form II RNA polymerase activity was quantitated by subtracting the c.p.m. incorporated in the presence of α -amanitin from those assays performed in the absence of α -amanitin. Nuclear forms I and III RNA polymerase activities were quantitated by subtracting the c.p.m. obtained from control incubated samples containing 80 $\mu\text{g ml}^{-1}$ actinomycin D plus α -amanitin (0.1 $\mu\text{g per assay}$) from the c.p.m. incorporated in the presence of α -amanitin alone. There is no linear incorporation of ³H-UMP into RNA in this control. Activities of RNA polymerases are expressed as pmol UMP incorporated per 5 min per mg DNA. DNA determinations were performed on identical 50- μ l aliquots of the same nuclear–nucleolar samples used for RNA polymerase assay by the method of Burton²³ using calf thymus DNA (Sigma, Type II) as a standard. Each point represents the average of five individual determinations $\pm$ s.e.m. Where error bars are not evident, they lie within the symbol. Control activities were: α -amanitin sensitive nucleoplasmic (form II RNA polymerase), 198; nuclear α -amanitin insensitive nucleoplasmic (forms I and III RNA polymerases), 114; and isolated nucleoli, 241 pmol UMP incorporated per 5 min per mg DNA, respectively. ●, Nucleoplasmic polymerase II; ○, nuclear RNA polymerases I and III (α -amanitin insensitive 0.1 $\mu\text{g per assay}$); and □, isolated nucleolar (RNA polymerase I) transcription. The arrow indicates 0.04 $\mu\text{g ml}^{-1}$ actinomycin D.

Is there any nucleoplasmic component of *in vitro* transcription in nuclei which is inhibited by actinomycin D? Briefly, nuclei and nucleoli were isolated from adult rat livers and resuspended in a sucrose buffer. Actinomycin D was preincubated with these organelles before the addition of 5-³H-UTP and substrates. Reactions were terminated by pipetting samples on to filter paper disks, washed, and counted as described in the legend to Fig. 1.

The effect of actinomycin D on the various RNA polymerase activities in isolated rat liver nuclei and purified nucleoli is shown in Fig. 1. Since actinomycin D inhibits rRNA transcription *in vivo* in the concentration range 0.001–0.1 $\mu\text{g ml}^{-1}$ (ref. 24), it was surprising to observe that transcription in isolated nucleoli was inhibited at concentrations almost 100 times greater. Transcription in isolated nuclei in the presence of α -amanitin shows near identical inhibition to that observed in nucleoli except that the curve is biphasic. This additional activity between 1 and 10 $\mu\text{g ml}^{-1}$ actinomycin D was inhibited by higher concentrations of α -amanitin (400 $\mu\text{g ml}^{-1}$)²⁵ and, therefore, confirmed to be RNA polymerase III (data not included). An additional experiment was performed to rule out the possibility that actinomycin D was not preincubated with isolated nucleoli for an adequate amount of time for sufficient inhibition to occur. Figure 2 presents this experiment in which isolated nucleoli were incubated at 30 °C in the presence of 0.04 and 0.1 $\mu\text{g ml}^{-1}$ actinomycin D relative to a control nucleolar sample incubated in the absence of actinomycin D. Samples of nucleoli were withdrawn at 15, 30, 45 and 60 min to test for the ability of the endogenous

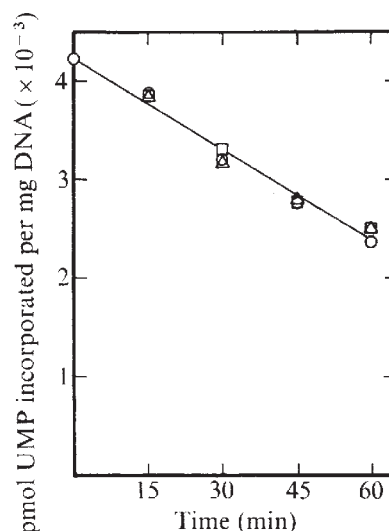


Fig. 2 Effect of actinomycin D preincubation with purified nucleoli on the activity of endogenous nucleolar RNA polymerase I. Actinomycin D at 0.04 and 0.1 $\mu\text{g ml}^{-1}$ were preincubated with identical nucleolar samples at 30 °C for varying times up to 60 min. Aliquots (50 μ l) were removed at the times indicated and pipetted into ³H-UTP and substrates for assay of endogenous nucleolar polymerase activity as described in the legend of Fig. 1. Results obtained with actinomycin D are plotted along with an identical nucleolar sample which contained no actinomycin D. ○, Control; △, 0.04 $\mu\text{g ml}^{-1}$ actinomycin D; and □, 0.1 $\mu\text{g ml}^{-1}$ actinomycin D. RNA polymerase activity is expressed as pmol UMP incorporated per 10 min per mg DNA.

RNA polymerase I to transcribe nucleolar DNA. This experiment demonstrated that actinomycin D is still ineffective in inhibiting transcription in isolated nucleoli after preincubation of these organelles up to 1 h. The loss of activity with time may be related to the spontaneous loss of RNA polymerase I activity as previously described by Lindell¹⁹ or to thermal inactivation of the enzyme.

Nucleoplasmic transcription by RNA polymerase II is biphasically inhibited by actinomycin D. Twenty per cent of the total nucleoplasmic RNA polymerase activity is inhibited by actinomycin D in the concentration range 0.001–0.1 $\mu\text{g ml}^{-1}$, whereas the remaining 80% is inhibited between 0.1 and 100 $\mu\text{g ml}^{-1}$. The first phase of nucleoplasmic polymerase transcription is inhibited at a concentration of actinomycin D 700 times lower than that required for the inhibition of the remaining nucleoplasmic activity.

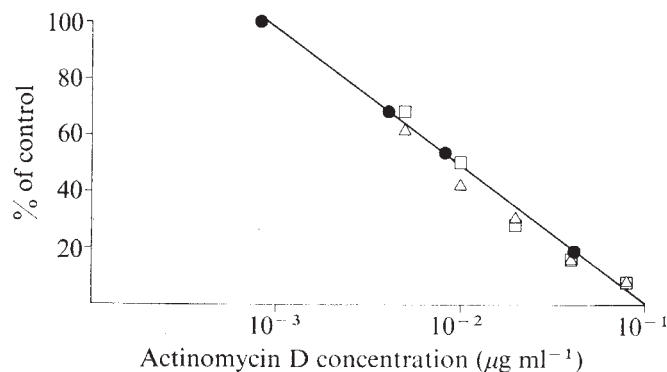


Fig. 3 Correlation of the inhibition of nucleoplasmic transcription *in vitro* with the inhibition of 45S RNA synthesis in mouse L cells *in vivo* (data of Perry and Kelley²⁴) by actinomycin between 0.001 and 0.08 $\mu\text{g ml}^{-1}$. The first phase of nucleoplasmic transcription (Fig. 1) was relativised to 100% and compared with the data of Perry and Kelley²⁴ (their Figs 4 and 7, 100 and 60 min preincubation of mouse L cells with actinomycin D, respectively). ●, Nucleoplasmic transcription (this study); □, Perry and Kelley, 60-min preincubation (their Fig. 7); and △, 100-min preincubation (their Fig. 4).

Although actinomycin D inhibition of *in vitro* transcription seems to be reversed with regard to nucleolar and nucleoplasmic RNA synthesis, there is at least one consistency with another observation of actinomycin D inhibition in cells in culture relative to this work. It is clear from the work of Perry and Kelley²⁶ that 4S and 5S RNA synthesis which is transcribed by RNA polymerase III (ref. 25), is 50–100-fold less sensitive to inhibition than 45S RNA transcription in mouse L cells. This is also apparent in this *in vitro* work but the concentration range of actinomycin D which affects 45S RNA transcription *in vivo* only affects nucleoplasmic RNA synthesis (presumptive mRNA). The relationship of this actinomycin D inhibition of nucleoplasmic transcription in rat liver nuclei *in vitro* to the inhibition of 45S RNA synthesis in mouse L cells is shown in Fig. 3. It can be seen that an excellent correlation exists between these two heterologous observations.

Perhaps the most severe criticism of this work is that transcription in these organelles is not sufficiently sustained to allow any reasonable conclusions from a study such as this. Zylber and Penman²⁷ have concluded that transcription in HeLa cell nuclei is merely completion of synthesis which had already initiated *in vivo*. In fact, transcription in isolated rat liver nuclei assayed in these conditions (polymerase II and polymerases I+III) is only linear for about 5 min. Transcription in isolated nucleoli assayed in identical conditions is, however, linear for 30–45 min. In spite of the lack of sustained transcription in isolated nuclei, there is a reasonable correlation with the inhibition of nucleolar transcription and the activity of α -amanitin-insensitive RNA polymerase activity in nuclei (Fig. 1).

The information presented in this manuscript lends further support to a possible extranucleolar control of rRNA transcription *in vivo*. Since actinomycin D only inhibits transcription by RNA polymerase II *in vitro* in the identical concentrations which inhibit 45S RNA transcription *in vivo*, it is likely that this extranucleolar control resides at the level of mRNA transcription. In fact, other investigators have observed an inhibition of nucleoplasmic RNA transcription by low concentrations of actinomycin D in eukaryotic cells in culture^{24,27,29}. The amount of nucleoplasmic RNA inhibited ranged from 5 to 50% depending on the cell type and the concentration of actinomycin D used.

If rRNA transcription is controlled by nucleoplasmic RNA polymerase II transcription (mRNA) as suggested by these data, then a rational hypothesis can be formulated with regard to the early observations of Fiume and Laschi⁷ regarding nucleolar fragmentation induced by α -amanitin. While inhibitory effects of α -amanitin on rRNA synthesis have been observed^{4–6}, others have seen no effect of this compound in specific cell types^{10,30}. It is, however, clear from these studies that α -amanitin probably does not easily penetrate the cell membrane because nucleolar fragmentation is not evident until 6–8 h after addition of α -amanitin (5 $\mu\text{g ml}^{-1}$) to CHO cells and effects on rRNA synthesis are not observed until 24 h (ref. 10). For α -amanitin to be an effective inhibitor of rRNA transcription by the proposed pathway, all RNA polymerase II molecules must be inhibited to be assured that the expression of any specific genome is terminated. Actinomycin D, on the other hand, probably acts at a specific DNA site(s) which has a very high affinity for binding and, hence, a selective inhibitory action. These data would predict that that site is nucleoplasmic rather than nucleolar.

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Possible pathway for prebiotic uracil synthesis by photodehydrogenation

THE synthesis of purines in potentially prebiotic conditions has been demonstrated^{1,2}. Adenine and guanine have been synthesised directly from aqueous cyanide solutions and a mechanism for the concentration of hydrogen cyanide in low melting eutectics has been proposed³. These purines are also obtained from solutions of cyanogen (unpublished). The pyrimidines, however, have provided a more difficult problem. Uracil has been obtained from malic acid and urea, but only by heating in the presence of concentrated sulphuric acid⁴ or polyphosphoric acid⁵. Low yields of uracil have been inferred from spectral data for a reaction involving acrylonitrile, urea and ammonium chloride⁶. Further details concerning the conditions of this reaction, however, have not been offered. Cytosine and, indirectly uracil, have been prepared from cyanoacetylene and cyanate^{7,8}. The rapid hydrolysis of these reactants raises doubts about this route. Cyanoacetaldehyde, a hydrolysis product of cyanoacetylene, condenses with guanidine in aqueous solution to yield 2,4-diaminopyrimidine, which is hydrolysed to cytosine and uracil. The later route is considered to be more plausible⁹. Thymine has been prepared in 0.1% yield from uracil by a hydroxymethylation–reduction sequence involving formaldehyde and hydrazine hydrate in ammoniacal solution¹⁰. In view of uncertainties concerning the abundances on the primitive Earth of a number of the precursors which have been used in these studies, it seemed desirable to consider alternative routes to the synthesis of pyrimidines. In this report we describe the synthesis of uracil by the photodehydrogenation of 5,6-dihydrouracil (DHU), as well as the synthesis of DHU from β -alanine and urea in mild conditions.

β -Ureidopropionic acid (UPA), which is readily formed from β -alanine by reaction with urea¹¹ or with cyanate^{12,13}, is known to cyclise to DHU. The cyclisation is catalysed by acid¹², although certain substituted derivatives have been cyclised by heat¹⁴. We have found that the cyclisation of UPA can be affected by heating at moderate temperatures. UPA (2 g) in 20 ml H₂O was added to 25 g silica gel (Merck Kieselgel 60) and the water evaporated *in vacuo*. The mixture was then heated at 65 °C under N₂ for 5 weeks. DHU was isolated by column chromatography on cellulose in butan-2-ol–water (86 : 14) and characterised as *N*-acetyl-5,6-dihydrouracil (207 mg) by mixed melting point and infrared spectroscopy¹⁵. We have observed that the cyclisation of UPA to form DHU is promoted by urea. The direct evaporation of dilute solutions of β -alanine and urea either alone or in the presence of various silicates also results in the formation of DHU in the temperature range 60–90 °C. Further details of these experiments will be reported elsewhere.

In a typical photodehydrogenation experiment, 50 mg DHU in 5 ml hot distilled water was added to 1 g support material (Table 1), contained in a borosilicate tube (160 × 50 mm). The tube was equipped with inlet and outlet side arms for N₂ purging and a ground-glass joint for insertion of a Hanovia low-pressure mercury arc lamp. The tube was fitted to a rotary evaporator and the slurry evaporated and deposited on the walls of the tube *in vacuo*. Irradiation was carried out under N₂ for 48 h. The effect of water vapour on the reaction was tested by passing the N₂ through a flask filled with distilled water or, alternatively, through KOH. At the end of the irradiation period, the material was extracted with 100 ml hot distilled water and filtered. The products from these experiments gave

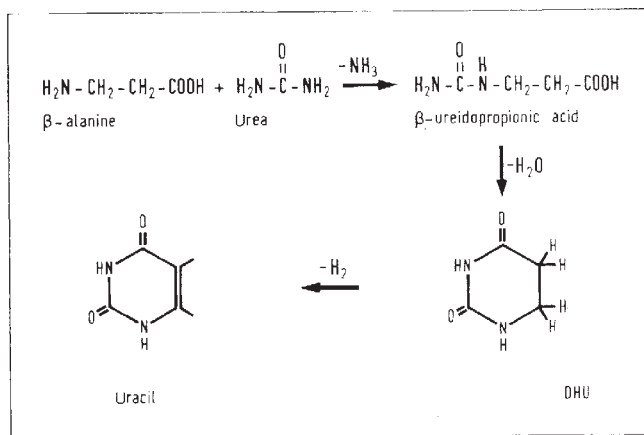


Fig. 1 Postulated pathway for prebiotic formation of uracil.

positive Wheeler–Johnson tests¹⁶. The presence of uracil was confirmed by ultraviolet absorption spectroscopy and by thin-layer chromatography on silica gel (*n*-butanol–acetic acid–water; 3 : 3 : 1) and on cellulose (*tert*-butanol–methyl ethyl ketone–water–concentrated NH₃, 40 : 30 : 20 : 10). Products from a number of experiments were pooled, and uracil was isolated by preparative thin-layer chromatography on cellulose as described above. Acetylation gave *N*-acetyluracil, as shown by comparison with an authentic sample by infrared spectroscopy and mixed melting point¹⁵. Quantitative analysis of uracil was carried out by ion-exclusion chromatography on Aminex A25 and A6 columns as described previously^{17,18}.

Results are summarised in Table 1. In the absence of added support, 2% conversion of DHU to uracil was obtained after

Table 1 Conversion of 5,6-dihydrouracil to uracil on various supports

Support*	Yield of uracil (mole %) [†]
Montmorillonite number 20	22 (6)
Montmorillonite number 23	14 (7)
Montmorillonite number 31	12 (5)
Illite number 35	5 (2)
Illite number 36	4
Vermiculite (Zonolite)	3
Kaolinite number 9	2
Kaolinite number 5	1
Silica gel	3 (2)
Quartz	2
Basalt	2
Granite	1
CaCO ₃	2
None	2 (1)

*CaCO₃ and silica gel (Kieselgel H) were from Merck. Clay minerals were standard samples from Ward's and were hand ground. Quartz sand (Baker) and the basalt and granite samples were ground in a disk mill (Tema).

[†]Irradiation for 48 h under water-saturated N₂ as described in the text. Figures in parentheses are yields obtained with dry N₂.

48 h. In the presence of montmorillonite number 20, however, the yield of uracil was 22%. This difference, as well as the influence of water vapour on the yields, suggests that the most active materials exert an effect by binding water in the solid phase and perhaps catalysing its photolytic dissociation. (Irradiation of DHU in aqueous solution also yielded uracil. After 4 d a 7% yield was obtained from a 0.0044 M solution.) Destruction of starting material and/or product was also more severe in the presence of water vapour. Thus, in the experiments with montmorillonite, uracil+DHU accounted for 90 to 97% of the starting material in the absence of water vapour, but only 74 to 82% in its presence. The series—montmorillonite, illite, vermiculite, kaolinite—is particularly interesting, as it represents a transition from expandable lattice structure

(montmorillonite) to non-expandable (kaolinite)¹⁹. A plausible mechanism for the photodehydrogenation might involve dissociation of bound water molecules and the hydroxylation and subsequent dehydration of DHU. The mechanism may therefore be similar to that involved in the ⁶⁰Co γ radiolysis of DHU, in which 5-hydroxy-, 6-hydroxy- and 5,6-dihydroxy DHU are formed²⁰. Studies on the γ radiolysis of dihydrothymine have also implicated the OH radical as the reactive species in the formation of thymine²¹. Our studies on the mechanism of the catalysed photodehydrogenation are continuing.

We suggest that the synthesis of uracil by the reaction sequence shown in Fig. 1 represents a reasonable prebiotic pathway. Urea has been repeatedly suggested as a prebiotic reagent²². As the product formed in highest yield from solutions of hydrogen cyanide as well as by other pathways, it is one of the most plausible nitrogen-containing prebiotic materials and has been proposed as a possible storage form for ammonia on the primitive Earth²³. β -Alanine has been reported in the Murchison meteorite²⁴, and has been formed in a number of simulated prebiotic syntheses²⁵⁻²⁷. Its abundance relative to other amino acids varies depending on the conditions chosen. In our proposed pathway, β -alanine would probably have been limiting. The entire pathway of carbamylation, cyclisation and photodehydrogenation may have occurred in evaporating pond environments, particularly where cycles of dehydration and rehydration were common.

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duction after homogenisation. On the other hand, ethane is produced as the main hydrocarbon gas after homogenisation of certain tissues of higher plants^{1,2}. We have investigated the influence of point freezing on ethylene and ethane synthesis by sugar beet leaf disks, and found that both are stimulated.

Rates of ethylene and ethane formation were monitored after incubation of untreated or point frozen leaf disks of 23 mm diameter and about 100 mg fresh weight, in order to show that sugar beet leaf tissue behaves as theoretically expected^{1,2}. The disks were cut from the upper third of sugar beet leaves not more than 12 weeks old, and floated on 1 ml of water in 3-ml Fernbach flasks, closed by a serum rubber stopper. After 2 h of incubation at 25 °C in the dark, 0.5 ml of the gas phase was analysed for ethylene and ethane by gas chromatography (compared with authentic gases purchased by Messer-Griesheim GmbH, Dusseldorf) as described before³.

Table 1 Ethylene and ethane production of untreated and pressed sugar beet leaf disks

	Ethylene (a) (pmol per h per g fresh weight)	Ethane (b) (pmol per h per g fresh weight)	Ratio a/b
Untreated disks	379 $\pm$ 355 (61)	1 $\pm$ 3 (35)	95 $\pm$ 320 (35)
Pressed disks	33 $\pm$ 171 (41)	86 $\pm$ 40 (60)	0.4 $\pm$ 2.6 (41)

Results are mean $\pm$ s.d. Figures in parentheses are numbers of experiments. These data were compared by statistical methods (*t* test, *F* test). Rate of ethylene production of untreated disks > pressed disks ($P < 0.01$). Rate of ethane production of pressed disks > untreated disks ($P < 0.01$). Ratio a/b of untreated disks > pressed disks ($P < 0.01$).

The most ethylene was formed by untreated leaf disks (Table 1). Production of ethylene was strongly reduced after the leaf disks had been pressed with a plastic piston on a plastic board. In contrast, ethane production was low in untreated leaf disks and was strongly stimulated after pressing. These results, obtained by pressing sugar beet leaf disks, are in good agreement with results obtained after homogenisation of various higher plant tissues^{1,2}.

The following experiments demonstrate a correlation between the extent of wounding and the rates of production of ethylene and ethane by sugar beet leaf disks. For this purpose, leaf disks of 5-week-old sugar beets were frozen to various extents: a stainless steel rod, 3 mm in diameter, kept at the temperature of liquid nitrogen, was touched to the surface of the leaf disks for 2 s. This resulted in a frozen circular area of approximately 3% of the total leaf disk area. By repeating this process, leaf disks were point frozen to 25, 50, 75 and 100% with respect to the total leaf disk area and were then incubated as described above. Figure 1 shows the rates of ethylene and ethane production plotted against the percentage of frozen leaf disk areas. Production of ethylene increased as the extent of point freezing of the total leaf disk area increased from 0 to 50%, and decreased linearly as the frozen leaf disk area increased from 50 to 100%.

In contrast to ethylene, ethane formation increased linearly as the point freezing of the total leaf disk area increased from 0 to 100% (Fig. 1). The measured ethylene to ethane ratios plotted against the percentage of frozen leaf disk area (Fig. 2) shows a strict logarithmic dependence.

The linear stimulation of ethane production after leaf disk point freezing up to 100% of the disk area indicates that, in contrast to ethylene, ethane production is not dependent on intact compartmentalisation in the leaf cells, but rather depends on cellular disorder. Thus, ethane production and the ethylene to ethane ratio can be taken as an indicator of the integrity of sugar beet leaf tissue.

Effect of point freezing on ethylene and ethane production by sugar beet leaf disks

THE site of biosynthesis of the plant hormone ethylene in the cells of higher plants is unknown. Efforts to identify the enzyme system responsible and its subcellular location have failed because ethylene-producing plant tissues stop pro-

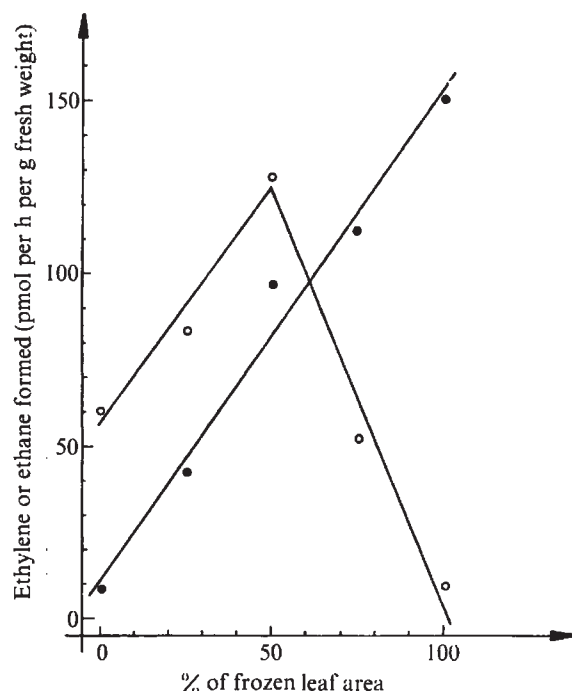


Fig. 1 Dependence of ethylene (○) and ethane (●) production on the percentage of frozen leaf area. Point freezing and incubation were achieved as described in the text. The points of the lines represent the mean of five experiments.

The influence of freezing on ethylene production seems to be more complex. Up to a certain extent of point freezing (in our case 50% of frozen leaf disk area), ethylene production increased after point freezing in spite of the decrease of unfrozen leaf area. When 50% of leaf disk area was frozen, further point freezing severely decreased ethylene production to an almost negligible amount. Assuming a K_m for O_2 of 0.2% for ethylene synthesis⁴, the decrease of ethylene production and the concomitant increase of ethane production cannot be attributed to oxygen depletion as the result of an increased oxygen consumption after point freezing. This can be concluded from the observation that pressed leaf disks of 100 mg fresh weight consume about $1.6 \mu\text{mol}$ of O_2 per 2 h,

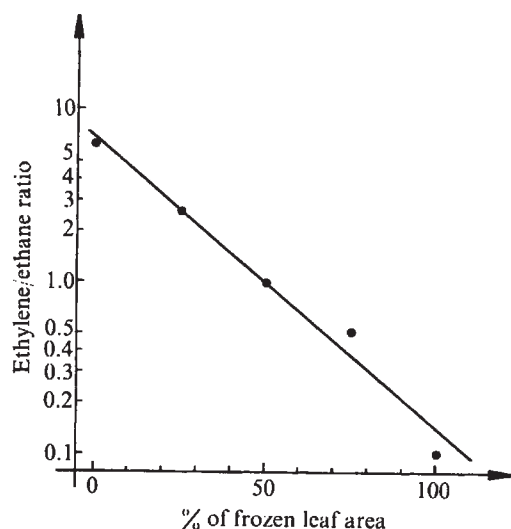


Fig. 2 Dependence of the ethylene to ethane ratio on the percentage of frozen leaf area. Point freezing and incubation were achieved as described in the text. The points of the lines represent the mean of five experiments.

representing 8% of the total amount ($20.0 \mu\text{mol}$) of O_2 available. From the above results we conclude that ethylene formation—in contrast to ethane formation—occurs in the leaf area surrounding the frozen parts of the leaf, representing a wound response of the non-departmentalised, but physiologically perturbed^{5,6} cells, adjacent to the departmentalised (killed) cells.

If the percentage of the departmentalised tissue was increased, the non-departmentalised, but perturbed tissue was reduced, this being observed as a decrease in ethylene production.

The physiological role of the stimulation of ethylene production after wounding can be seen in the induction of wound responses, for example, the accumulation of phenolic compounds⁷. It is thus interesting that injury-related biosynthesis of an aromatic phytoalexin (phaseollin) in *Phaseolus vulgaris* has also been described to be dependent on living tissue adjacent to the injuries⁸. Ethane production, on the other hand, does not depend on intact tissue but rather on cellular disorder and loss of compartmentalisation, for the highest production rates are obtained with 100% frozen leaf area. Because of this independent behaviour of ethylene and ethane production, two different pathways can be assumed for their biosynthesis.

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Errata

In the article "Significance of impulse activity in the transformation of skeletal muscle type" by S. Salmons and F. A. Sreter (*Nature*, **263**, 30; 1976), the tenth line from the bottom of the first column on page 33 should read . . . soleus muscles which had received stimulation showed light- . . . and not as printed.

In the article "Opposing effects of cyclic AMP and cyclic GMP on protein phosphorylation in tubulin preparations" by I. V. Sandoval and P. Cuatrecasas (*Nature*, **262**, 511; 1976) the pH value given in the second line of the legend to Table 1 should read pH 6.75 and not 7.65 as printed.

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matters arising

Green rust: a pyroaurite type structure

MCGILL, MCENANEY AND SMITH¹ have described a green corrosion product of iron developed in water containing NaCl, K₂SO₄, NaCO₃, mixtures of these salts, and NaOH under various degrees of aeration. The X-ray data they reported indicate that the product is a member of the pyroaurite group²⁻⁴. In fact, Stampfl⁵ and Taylor⁶ have also concluded that a ferrous-ferric green rust material belongs to the group of pyroaurite structures.

We have been concerned⁷ with the synthesis of products similar to the mineral takovite which has a formula of the pyroaurite type near to (Ni,Mg)₆(Al,Fe³⁺)₂(OH)₁₆CO₃·4H₂O. We find that such products are formed readily in solutions containing Cl⁻, NO₃⁻ and SO₄²⁻ ions, provided that CO₂ is not rigorously excluded. When water in its normal state or saturated with CO₂ is used, the same or similar final product is obtained. The presence of the anion CO₃²⁻ is shown unambiguously by infrared spectroscopic data. We suspect that other anions play only a small role in the formation of the product when CO₃²⁻ ions are present. For reasons which are not entirely clear, the pyroaurite structure type seems to form very readily when CO₃²⁻ ions are available. Possibly the planar nature of the CO₃²⁻ ion and the total number required are favourable factors; NO₃⁻ ions, although planar, must be twice as numerous for electrical neutrality.

Using the X-ray data reported by McGill, *et al.*¹, we have recalculated the unit cell parameters using the refinement program of Evans *et al.*⁷; the results are: $a = 3.166 \pm 0.001$, and $c = 22.52 \pm 0.01$ Å—values which are close to those given by McGill *et al.* and also by Stampfl⁵: $a = 3.17$; $c = 22.8$ Å.

It is of interest to consider a possible relationship between cation composition (as indicated by the weighted mean cation radius, $\bar{r}$), and the hexagonal a parameter. Figure 1 shows such a plot with data taken from the literature, including two unpublished points (G.W.B. and D.L.B.), dealing with takovite. The $\bar{r}$ values are calculated using the crystal radii of Shannon and Prewitt⁸. The smallest value of $\bar{r}$ is for takovite (G.W.B. and D.L.B., unpublished) and the largest for the green rust studied by Butler and Benyon⁹ on the assumption of a 3 : 1 ferrous-ferric ratio. The available data cluster near to a

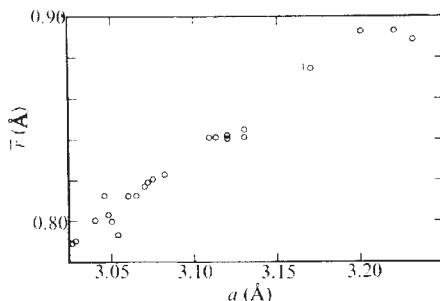


Fig. 1 Plot of $\bar{r}$ (mean cation radius), against unit cell parameter a for pyroaurite-type structures. Vertical line indicates the a parameter of the green rust of McGill *et al.*¹.

straight line and indicate for the green rust of McGill *et al.* a ferrous-ferric ratio of about 2:1; the precise result depends on how the line is drawn. In future studies of pyroaurite group materials, it will be useful to have chemically analysed materials and accurate cell parameters so that a composition-cell parameter graph can be drawn with greater accuracy than is now possible.

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AN extraordinary orientational relationship between the rhombohedral green rust (with hexagonal unit cell dimensions $a = 3.181$ Å and $c = 21.82$ Å) and magnetite (cubic with $a = 8.434$ Å)—namely, $[1\bar{1}\bar{1}]_{\text{rh}} // [42\bar{1}]_{\text{hex}} // [1\bar{1}\bar{1}]_{\text{cub}}$; and $(566)_{\text{rh}} // (1\ 0\ \bar{1}\ 17)_{\text{hex}} // (111)_{\text{cub}}$ —has been reported by McGill, McEnaney and Smith¹. I should like to query this result, and to suggest that there is really no evidence that the relationship is other than $[00.1]_{\text{hex}} // [111]_{\text{rh}} // 111_{\text{cub}}$, and $(30\bar{3}0)_{\text{hex}}$

or $(2\bar{1}\bar{1})_{\text{rh}} // (112)_{\text{cub}}$, as would be expected from a comparison of the oxygen packings of the two structures.

If the material is not well crystallised, or the platelet is very thin, the reciprocal lattice points will be extended parallel to $[00.1]_{\text{hex}}$ and it will not then be necessary to postulate that the diffraction spots, visible in their Fig. 1 and obtained when the beam is perpendicular to the crystal flake, have indices which strictly obey the requirement $-h+k+l = 3n$. The photographs reproduced in ref. 1 do not seem to show departures from hexagonal symmetry. Moreover, the hexagonal and rhombohedral cell dimensions are not entirely consistent with each other and the sets of planes considered to be equivalent do not really have similar d -spacings.

The simpler topotactic relationship had been reported earlier^{2,3} but, together with work on the structure of the green rust materials⁴, had gained inadequate publicity.

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MCGILL ET AL. REPLY—Mackay's argument¹ presumes that there is close matching of oxygen ions in the green rust and magnetite structures resulting from a topotactic transformation, that is, internal atomic displacements resulting in accord in three dimensions between initial and final lattices². There is no conclusive evidence for this type of transformation from our experiments on the corrosion of cast iron. It has been found that magnetite and green rust usually precipitate from solution independently of each other, although there are isolated examples of the epitaxial growth of magnetite on green rust from solution. Figure 3 of our original contribution³ illustrates this, and shows magnetite as three-dimensional, 'island' outgrowths from the surface of green rust crystals, a morphology commonly observed in epitaxy. Close matching of oxygen ions between green rust and magnetite is not, therefore, a necessary condition, since epitaxy can occur with large degrees of atomic

mismatch between substrate and overgrowth⁴.

The orientation relationship that we report results from a correlation of *d*-spacings from electron diffraction, and the more accurate *d*-spacings from X-ray diffraction; such a correlation cannot be obtained if the orientation relationship proposed by Mackay¹ is assumed. Nor is there evidence to postulate a relaxation in the Laue condition, since scanning electron microscopy (see Fig. 3, ref. 3) indicates that the green rust crystals are $\sim 0.1 \mu\text{m}$ in thickness or greater. The elongation of diffraction spots is usually associated with microcrystals which have critical dimensions of much less than $0.1 \mu\text{m}$. Electron diffraction suggests that the green rust platelets are well-crystallised. An inspection of the rhombohedral unit cell shows that a flat crystal with a $(\bar{5} \bar{6} \bar{6})$ habit plane may have hexagonal geometry.

Turning to the point raised by Brindley and Bish⁵: in our experiments green rust is grown in closed vessels by the corrosion of cast iron in solutions which are deoxygenated by purging with nitrogen. Thus the level of dissolved carbon dioxide is very low in non-carbonate solutions. For example, green rust and magnetite were formed by the corrosion of cast iron at 50°C in distilled, deionised water containing 0.4 p.p.m. of oxygen. Chemical analysis of the water gave the following results: before corrosion, $\text{pH} = 7.4$, alkalinity = 5.0 p.p.m.; after corrosion, $\text{pH} = 6.6$, alkalinity = 5.5 p.p.m.; alkalinity = $[\text{CO}_3^{2-}] + [\text{HCO}_3^-] + [\text{CO}_2] + [\text{OH}^-]$. Since, for H_2CO_3 at 50°C , $K_1 = 5.19 \times 10^{-7}$ and $K_2 = 6.73 \times 10^{-11}$ (ref. 6) the bicarbonate ion is the predominant species in this pH range, and the carbonate ion concentration is extremely low.

In another experiment, green rust produced in sodium carbonate solution, $[\text{CO}_3^{2-}] = 200$ p.p.m. was examined using infrared spectroscopy. The presence of the hydroxyl group was clearly shown but the carbonate ion was not detected. In view of the comments of Brindley and Bish⁵, a more extensive infrared spectroscopic investigation is being undertaken, but at present, our experiments do not support the view that the presence of the carbonate ion is particularly significant in the formation of green rusts by the corrosion of cast iron.

These and other points relating to processes involved in corrosion of cast iron will be developed elsewhere.

Production of heavy elements in neutron stars

CHECHETKIN and Kowalski¹ have proposed that the production of heavy elements in nature occurs by the ejection of matter from a neutron star surface. Their suggestion relies on the results of Chechetkin and Bisnovatyi-Kogan^{2,3}, who find such matter to be composed before ejection of 'jumbo' nuclei ($Z \gtrsim 160$; $A \gtrsim 640$). These results were derived using a highly uncertain extrapolation of binding energies into the extreme neutron-rich region and in the absence of fission. Their justification of the neglect of fission is based on a necessarily very crude estimate of fission lifetimes.

The nuclear stability against spontaneous fission is the result of a barrier in the potential energy curve of a nucleus as a function of deformation. In the framework of the macroscopic-microscopic method^{4,5}, the potential energy can be divided into two parts: (1) a macroscopic contribution that takes into account the smooth variation of the energy as a function of nucleon number and deformation, and (2) a microscopic contribution that includes shell effects which are significant only near the magic numbers for neutrons or protons. The macroscopic energy is calculated using the liquid-drop or droplet model of the nucleus, whereas the microscopic contribution is evaluated from the energy spectrum associated with an appropriate potential well.

As the proton number and the neutron excess of the nucleus are increased, the stability against fission resulting from the macroscopic energy is reduced both by the increased repulsion of the Coulomb force with the addition of protons and by the decreased surface tension of the nucleus with the addition of neutrons. It is the rapid decrease of the macroscopic energy as a function of deformation that causes nuclei beyond the actinide region ($A \gtrsim 260$) to be unstable with respect to spontaneous fission. Beyond the actinide region, only strong single-particle effects near neutron or proton closed shells (as for the case of the superheavy island $A \simeq 298$, $N \simeq 184$, $Z \simeq 114$) can offer stability against spontaneous fission. In the immediate region surrounding the superheavy island, the fission barriers of nuclei are virtually non-existent⁶.

Furthermore, in a neutron capture the neutron separation energy in the compound nucleus plus the kinetic energy of the incoming neutron easily lead to subsequent fission. Consequently, any process of successive neutron captures will be terminated by neutron-induced fission before significant spontaneous fission can occur. Recent calculations^{6,7} of fission barrier heights and neutron separation energies in the *r*-process region indicate that the astrophysical *r* process

may well be terminated by neutron-induced fission in the mass region $A \sim 280$. It should, however, be pointed out that the exact point of this termination is very uncertain because of an extrapolation of surface-asymmetry and closed-shell effects from the region of known nuclei and because of the use of the Strutinsky procedure. The termination point may be somewhat higher ($A \sim 350$), consistent with the possible discovery of superheavy elements in pleochroic halo inclusions⁸ and their production in a conventional *r* process⁹. In spite of the uncertainties of the calculations of fission barriers^{6,7}, we believe that the existence of nuclei as large as $A = 640$, $N = 480$, $Z = 160$ is definitely ruled out. We should also mention that similarly large nuclei had been predicted in equilibrium conditions in neutron star matter^{10,11}. This, however, has also been conclusively shown to be incorrect¹²⁻¹⁴. In conclusion, the nuclear basis of Chechetkin and Kowalski's suggestion for the production of superheavy elements is unfounded although it may well be that these elements can be produced in an *r* process occurring in the surface material of neutron stars. Studies along these lines are under way.

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reviews

Assuming the mantle of soothsayer

L. Knopoff

Earthquake Prediction. (Developments in Solid Earth Geophysics, Vol. 9.) By Tsuneji Rikitake. Pp. xvi+357. (Elsevier Scientific: Amsterdam, Oxford and New York, 1976.) Dfl.95; \$37.95.

STRONG earthquakes hold such terror for unprotected populations that those capable of forecasting earthquakes with any degree of reliability whatsoever, have a ready and appreciative audience. Thus, earthquake prediction has been the business of psychics and astrologers for centuries. The unhappy consequences of recent severe earthquakes have inspired significant effort in four nations to spur science to assume the mantle of soothsayer. Government-sponsored prediction research has been undertaken on a major scale in the USSR (since 1949), Japan (1963), China (1966) and the US (1971). In these countries, concerted efforts have been made in what must be the first priority in any programme—obtaining base-line information. These activities have been carried out to varying degrees in each country by measurement of strains, geodetic measurements, observations of micro-earthquake activity, geomagnetic, geoelectric and elastic properties, and collection of geochemical information.

Each of the four programs has its own emphasis. Professor Rikitake has been a leader of the Japanese program, and writes as an authority eminently qualified to review the Japanese program. The longest chapter in the book (62 pages) is concerned with geodetic surveying methods for observing precursory deformation of the Earth's surface and summaries of observations of such deformation before a number of earthquake events. Although geodetic observations are comparatively lightly emphasised in the other programs, they are a cornerstone of the Japanese national program.

The reader should not expect to receive a broad exposure to the basic problems of earthquake prediction or to the arguments and uncertainties that pervade the subject. Instead, this book provides a thorough review of

the phenomenology of observations deemed pertinent to prediction. The reader who expects to derive understanding of the processes that underly the observations should look elsewhere.

The physical basis of earthquake prediction is unsatisfactorily treated. The shortest chapter in the book (4 pages) is concerned with the dilatancy model of the focal zone; in this model, the volume of matter increases due to the formation of small cracks shortly before rupture. Dilatancy is the only physical model proposed so far that has the promise of providing an understanding for the various precursory phenomena. Professor Rikitake withdraws from a detailed discussion of dilatancy, including the controversy over the role of water as an active agent in the dilatancy process.

The lengthy chapter entitled "The Theory of Earthquake Prediction", has no theory therein, but is concerned instead with statistical approaches to earthquake prediction plus an exten-

sive catalogue of the events for which precursors have been observed. Two or three pages of the book are devoted to the phenomenology of brittle fracture and strike-slip processes; nothing is presented describing the physical basis in fracture mechanics for the earthquake event.

The book is well written in language understandable to the non-expert. The non-idiomatic use of the English language is not offensive. The editing and the selection of topics make the book a highly personal view of earthquake prediction. Major aspects of the topic deserve to be presented more broadly than is done here. In spite of extraordinary omissions, this book is useful for the extensive tabulations of observations of diverse precursory phenomena. □

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Magnetic resonance techniques

Magnetic Resonance of Biomolecules: An Introduction to the Theory and Practice of NMR and ESR in Biological Systems. By P. F. Knowles, D. Marsh and H. W. E. Rattle. Pp. 343. (Wiley-Interscience: New York and London, May, 1976.) Cloth £9.75, \$19.75; paper £4.25, \$8.75.

THE applications of magnetic resonance techniques to biological problems are now of sufficient importance to merit inclusion in any well-balanced undergraduate degree course in biochemistry or biophysics. Although there are several specialist books dealing with such applications there has been no text written primarily for the undergraduate student.

Drs Knowles, Marsh and Rattle have now produced such a book dealing with the applications of nuclear magnetic resonance (NMR) and electron spin resonance (ESR) spectroscopy to biological problems. On the whole, they have produced a very useful book for both student and teacher alike. The parts of the book dealing with ESR are particularly

valuable because such teaching material is not easily accessible elsewhere.

The basic theory of NMR and ESR are discussed together in an opening chapter and other chapters deal with the spectral parameters, practical aspects and biological applications of each technique. The carefully selected material is presented in an interesting manner and due emphasis is placed on the advanced experimental techniques which are required for successful biological studies. In general the theoretical aspects have been dealt with adequately although I would have preferred to see the theory of NMR and ESR introduced separately. In the NMR section a more detailed account of chemical exchange and relaxation behaviour would have been useful in view of the importance of these aspects of the theory in biological studies.

In some of the examples it was not always clear how the biological information had been derived from the NMR data. For example, a conformational structure for oxytocin was presented but none of the NMR argu-

ments leading to this were given. Obviously in a text of this type the authors have had to be very selective in their choice of examples of the numerous applications; they have succeeded admirably in finding examples which illustrate the scope of the techniques as well as providing interesting biological information.

The text would have been improved by including some consideration of conformational studies of small molecules of biological interest using spin-spin coupling constants and some studies of ligand binding to proteins. In general however the authors have provided an impressive coverage of the major areas of application including studies of protein and transfer RNA conformations and structures, protein unfolding, protein-protein interactions, protein hydration, metalloproteins, spin-labelled proteins and studies of phospholipid membranes.

This book can certainly be recommended to students and teachers wishing to learn more about the usefulness of magnetic resonance techniques in biology. **J. Feeney**

Dr Feeney is a member of the scientific staff in the Division of Molecular Pharmacology at the National Institute of Medical Research, London, UK.

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Immunology of reproduction

Immunobiology of Trophoblast. (Clinical and Experimental Immunoreproduction, 1.) Edited by R. G. Edwards, C. W. S. Howe and M. H. Johnson. Pp. x+284. (Cambridge University: Cambridge, London and New York, August 1975.) £6.

THIS volume represents the papers and discussion of a meeting held in January 1974 in the Physiology Laboratory, Cambridge, UK, and is the first of a series of meetings held on specific topics of immunoreproduction in association with the activities of the International Coordination Committee for the Immunology of Reproduction. All the participants were either British or were visiting Britain.

The papers all, in one way or another, address themselves to the key fact that the foetus is able to survive in the uterus in spite of the antigenic disparity which by rights should lead to its rejection as an allograft. Papers by A. C. Allison, by M. H. Johnson, by K. D. Bagshaw and S. Lawler, and by W. P. Faulk *et al.* document the complex antigenicity of the developing trophoblast, placenta and foetus. Details of trophoblast differentiation are described by R. L. Gardner and by W. D. Billington. The trophoblast and placental function in prevention of cell traffic and transfer of antigens and antibody is discussed by M. Adinolfi, whereas endocrine effects on the immunological consequences of implantation of the antigenically foreign foetus are described by R. Borland *et al.* W. R. Allen reports on the complex endocrinological and immunological features accompanying implantation and foetal development in the mare. Two extensive papers, one an overview of lymphocyte physiology by C. W. S. Howe, the other a review of the complex and often divergent observation on the effect of antigenic disparity on placental size, implantation and embryonic survival of mammalian embryos by A. McLaren complete the volume.

It is remarkable that in spite of the limited number of papers, the volume represents well the current state of knowledge in this field. The more comprehensive papers are doubtlessly the most valuable in that they focus in depth on the problems of trophoblast immunobiology, and in depth consideration of such problems is a luxury permitted all too infrequently in a time of increasing page charges and decreasing page allowances. The additional feature of publishing the discussion comments following each paper is especially

welcome, for it provides the reader with a feeling for the current state of acceptance of or disagreement with the views expressed by the authors.

The volume is a most valuable addition to the literature in the fields of reproductive biology and of immunology. **Robert Auerbach**

Robert Auerbach is Professor of Zoology at the University of Wisconsin, Madison, Wisconsin.

Membrane series

Biological Membranes. Vol. 3. Edited by Dennis Chapman and Donald F. H. Wallach. Pp. x+362. (Academic: London and New York, May 1976.) £11; \$27.75.

WHEN *Biological Membranes, Physical Fact and Function* was published in 1968 there was no indication that it was to become volume 1 of a series which has now reached volume 3 and seems likely to continue. The publication of such volumes at irregular intervals of several years tends to delay some of the contributions unfairly and to provide an irregular and unpredictable coverage of the field. Nevertheless, in the book under review, the editors have collected together a group of six interesting topics and appropriate authors, most of whom seem to have written their contributions during 1974. The chapters are, in general, authoritative and well presented, and on such a variety of topics as to provide something of interest for everyone. Most libraries and many individuals may therefore be persuaded to continue to collect the series.

Two of the new topics, concerned with neutron diffraction and scattering and with the use of lanthanides to probe the role of calcium in membranes, are still at the exploratory stage and are reviewed largely to emphasise their potential for membrane studies. Other chapters discuss lipid and protein mobilities in membranes and the exchange of lipids between membranes and lipoproteins. The two remaining chapters provide a comprehensive review of nuclear membrane structure and function, and a specialist assessment of the specificity and significance of interactions between plasma membranes and gammaglobulins. The author index is four times as long as the subject index even though the latter includes all but three of the rare earth elements (all listed on p154). **J. B. Finean**

Dr Finean is a Reader in Molecular Biology in the Department of Biochemistry at the University of Birmingham, UK.

Light scattering

Dynamic Light Scattering. By Bruce J. Berne and Robert Pecora. (Wiley: New York, March 1976.) £14.00; \$27.70.

CLOSELY following the introduction of the laser we have in recent years witnessed a considerable increase in the number of original publications and review articles on light scattering theory and its applications to chemistry, biology and physics. The novel feature which transformed classical light scattering into a modern field of study was the possibility of undertaking dynamic studies over an extensive range of time and frequency scales. Not all expectations have been fulfilled; the study of chemically reacting systems for instance has been rather disappointing so far and the successful extraction of higher order information, beyond the derivation of translational diffusion constants in dilute macromolecular solutions, has been the rare exception rather than the commonplace event.

From the book under review, as well as from its recent predecessor (see *Nature*, 255, 90, 1975) we learn that sufficient time has not yet elapsed to place the whole field into proper perspective. This reviewer would have been grateful for a logical development of modern scattering theory comprising the phenomenological, electromagnetic and molecular aspects. The authors are eminently qualified for this

undertaking. They present a welcome wealth of theoretical derivations, sometimes on a very elementary and at other times on a rather advanced specialised level. Interspersion of a large number of applications from many different fields detracts from the general purpose. Whereas the theory could easily have been presented as a permanent whole, the applications are fragmentary and ephemeral, and can only be fully appreciated by reference to the original works. The thread is broken and the consistency lost.

There are a number of details which I believe can be easily corrected in a future reprint. Some terms and names are misspelt, some references in the text are incomplete, and some references are given as 1974 and 1975 as being in press—in a book published in 1976. A glossary of symbols would have been helpful in view of the complicated nomenclature. To find out which diffusion coefficient is determined in the light scattering experiment the reader must struggle through the whole development of non-equilibrium thermodynamics taken from another source.

In spite of the above reservations, this book will be useful as a source-book of important theoretical information in specialised research laboratories active in a wide range of scattering studies.

Henryk Eisenberg

Henryk Eisenberg is a member of the Polymer Department, The Weizmann Institute of Science, Rehovot, Israel.

Pion-pion interaction

Pion-Pion Interactions in Particle Physics. By B. R. Martin, D. Morgan and G. Shaw. Pp. xi+460. (Academic: London and New York, May 1976.) £16; \$40.50.

AT some time during their career, most high energy physicists must obtain some acquaintance with the pion-pion interaction, because of its pervasive nature in hadronic physics. In future, I will have no hesitation in recommending the appropriate chapter of this book to them as an essential reference. The book provides a timely review of the subject, as there have been many important developments, both experimental and theoretical, since the appearance of Petersen's comprehensive *Physics Reports* article in 1971.

The lack of real pion targets means that special techniques have been developed to extract data on $\pi\pi$

scattering. A substantial part of this book is devoted to a careful explanation of these methods and the results from recent high precision experiments. The other major section of the book deals with theories and models of $\pi\pi$ scattering.

In places the book inevitably reflects the authors' own approach to the field. It is, for example, somewhat surprising that explicit expressions for the S and P wave contributions to the important Roy equations are not included in the otherwise extensive appendices. In general, however, they are to be congratulated on the care they have taken in preparing this book, which is destined to become a standard reference and should be available in all high energy physics libraries.

C. D. Froggatt

Dr C. D. Froggatt is a Lecturer in the Department of Natural Philosophy, Glasgow University and has made many contributions to the phenomenology of pion-pion scattering.

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obituary

Nikolai Ivanovich Muskhelishvili the eminent mathematician died on July 16. He was born on February 16, 1891 into a remarkable family, of great renown in Georgian history. His father, Ivan Levanovich, was a General in the Engineering Corps of the Imperial Russian Army, and had interesting ideas on the education of children. He spent a good deal of time teaching his own children—particularly 'Niko' whose mathematical ability he recognised at an early age. Muskhelishvili's mother, too, was a person of great culture as well as charm.

After completing his secondary education at the Second Tbilisi High School, Muskhelishvili entered the Faculty of Physics and Mathematics of the University of St. Petersburg in 1909. His outstanding ability early attracted attention, and legends were soon circulating among the student body of the accomplishments of the remarkably gifted young Georgian. He graduated in 1914 and in the following year presented a diploma thesis of such merit that he was invited to become research assistant to Gurii Vasil'evich Kolosov, a pioneer in the application of function theory to plane problems in elasticity. It was Kolosov who guided Muskhelishvili's first steps in the subject which in the next sixty years he was to revolutionise and dominate, and, in the process, to influence critically much of the development of mathematics (both pure and applied) in the USSR. One of his first papers, *Sur l'intégration de l'équation biharmonique*, published in 1919, gave a foretaste of the simplicity and elegance which were soon to become the distinguishing features of everything he wrote. As with most of his later writings on elasticity, the problem considered was of great practical as well as mathematical interest, for a special case considered by Muskhelishvili proved to be basic to the theory of brittle fracture in thin

plates.

In 1917 Muskhelishvili was appointed an assistant in what was now the University of Petrograd and two years later was promoted to an instructorship. It is typical of him that in the years 1915–1920 he gave a great deal of his time and energy to teaching in other institutions of higher learning in Petrograd which, because of the troubled times, were experiencing grave staffing problems.

When the university of Tbilisi was founded, he was invited to join the faculty. He returned to his homeland in 1920 and remained there until his death. Soon after his return to Georgia, Muskhelishvili assumed the role of leader of Georgian mathematics and created in Tbilisi one of the chief mathematical centres in the world. Initially, the group concentrated on the mathematical theory of elasticity, but later, comparable effort was put into important fields of analysis. Many of the famous names of Soviet mathematics appear in the records of the Tbilisi group and many more were influenced by the great stream of papers and research monographs which flowed from it.

Muskhelishvili's own research contributions were almost entirely in the theory of plane elasticity and in the theory of singular integral equations, but it was not only those branches of mathematics most directly connected with his own research interests that he sponsored. To take only one example: it was through his active support that the Georgian school of topology was founded and nurtured in its early years.

During this period of intense scientific activity extending over half a century, Muskhelishvili was tireless in his efforts to organise efficiently scientific activities in Georgia. He gave devoted service to the Academy of Sciences of the Georgian SSR, and from his election in 1939 as a full member of the

Academy of Sciences of the USSR he served repeatedly on its Presidium. When the National Committee of the USSR for Theoretical and Applied Mechanics was established in 1957 he was the obvious choice for its Chairman and he served long in that capacity, with the representation of the USSR on various international bodies which that office involves.

Muskhelishvili was influential in wider circles than the scientific. Believing that a university professor should participate fully in the life of his country he was active in the political field; among the offices he held was Deputy of the Supreme Soviet of the USSR.

For his scientific work and for the significant role he has played in the development of Soviet science, Muskhelishvili received many awards and honours, from his own government and from foreign institutions. Those who were privileged to know him and his family value the memory of his friendship and his gaiety, recall with affection the delights of his companionship as much as his incisive judgment on scientific questions. Future generations will know him only from his research papers and his two great monographs *Basic Problems in the Mathematical Theory of Elasticity* and *Singular Integral Equations* which achieve the seemingly incompatible aims of creating a satisfying unified theory and at the same time pointing the way to new realms of enquiry.

In all his published work Muskhelishvili showed that he was both a pure and an applied mathematician. The 'applied' aspect of his nature was shown by his willingness to look at problems of real practical significance, the 'pure' in the rigorous analysis to which he subjected the problems thrown up by his investigations into mechanics.

I. N. Sneddon

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